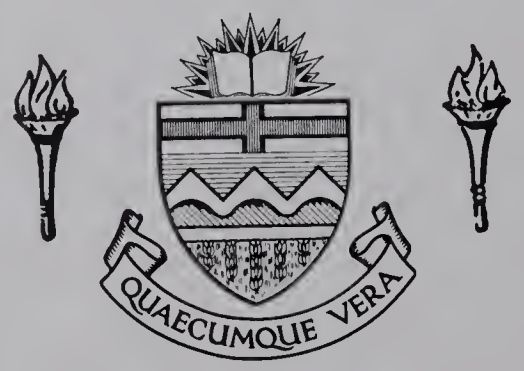


The University of Alberta
Printing Department
Edmonton, Alberta

THE UNIVERSITY OF ALBERTA

THE EFFECTS OF SEVERAL SPECTRAL BANDS OF VISIBLE LIGHT ON THE PHOTOSYNTHESIS OF AMINO ACIDS BY ISOLATED WAX BEAN CHLOROPLASTS

by

DEREK KURT TRACHSEL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN

PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

OCTOBER 1967

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

Thd undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Effects of Several Spectral Bands of Visible Light on the Photosynthesis of Amino Acids by Isolated Wax Bean Chloroplasts" submitted by Derek Kurt Trachsel in partial fulfilment of the requirements for the Degree of Master of Science.

ABSTRACT

The effect of light quality on photosynthesis of amino acids by chloroplasts was investigated. A series of filters and light assemblies was constructed and the light intensity of all the filters was adjusted to equal energies. Chloroplasts in a morphologically intact state were isolated from the leaves of Phaseolus vulgaris variety Kinghorn wax and purified on a discontinous sucrose density gradient. The purified chloroplasts when incubated with $^{14}CO_2$ under white light were found to incorporate twice as much label into the amino acids as the dark control. With ammonia present in the incubation mixture, the amount of label incorporated into the amino acids under the white light increased 15 times. When the chloroplasts were incubated under light of different wavebands, incorporation of label above that of the dark control occurred in the blue and red regions. However, the wavebands that were most effective in incorporation of label into individual soluble amino acids were not the same as those which were most effective for individual protein amino acids. This might indicate different pools of the same amino acid arising from different pathways of synthesis. Although absorption spectra of the separated pigments isolated from the purified chloroplasts were taken, no relation between any one pigment and the increase in the products of photosynthesis under a particular waveband could be made due to the rather

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Professor Saul Zalik for his guidance and help in many ways throughout the course of this research program and during the preparation of this manuscript.

Also, I would like to extend many thanks to Mrs. Ruth Craig for her assistance in many of the preparations during the course of experimental work.

Thanks are also extended to Mr. Michael Batory for his assistance in the analysis of the samples.

Financial assistance from the National Research Council of Canada is acknowledged with thanks.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Light is recognized as an important factor in the growth and development of plants. In addition to supplying the energy for photosynthesis, it controls certain morphogenetic effects. The organelle responsible for the conversion and utilization of light energy for the reduction of absorbed carbon is the chloroplast. Within the last few years, several workers have obtained data to indicate that light quality affects the distribution of absorbed carbon among the various products of photosynthesis (3, 12, 22, 32). Also, studies in this laboratory dealing with effects of light quality on seedling development (1, 18, 37) indicated an effect of light on metabolism and on pigment synthesis. The materials generally used to study these effects of light quality were intact seedlings, whole leaves or leaf discs and algae. Little if any work on the absorption of carbon under different wavebands by isolated chloroplasts has been reported.

To study the effects of light of different quality on the incorporation of ^{14}C from $^{14}CO_2$ into amino acids by chloroplasts, a homogenous purified chloroplast suspension from higher plants capable of photosynthesis without added cofactors is desirable. The use of such purified preparations would eliminate the metabolic effects of other organelles and permit characterization of the metabolism of the chloroplast as an independent unit.

This investigation was designed to adapt a method for the isolation of purified chloroplasts capable of incorporation of $^{14}CO_2$ into amino acids and to determine the effect of light of different quality on this photosynthesis. In order to relate the effects of light quality to the observed differences in labeling of certain amino acids the pigments from the purified chloroplasts were isolated and separated.

LITERATURE REVIEW

Photosynthesis in green plants requires three photochemical reactions, each representing an increasingly complex phase of photosynthesis. They are the photolysis of water, the esterification of inorganic phosphate to ATP, and the reduction of CO_2. The three photochemical reactions were found to be separable (4). Inhibition of a more complex phase of photosynthesis does not affect the simpler one which preceeds it. Conversely, the inhibition of the simpler phase of photosynthesis is paralleled by an inhibition of the more complex phase which follows. The third phase, the reduction of CO_2, is dependent on the two simpler phases of photosynthesis, ATP formation and photolysis. Jensen and Bassham (29) were successful in preparing a well defined homogenous suspension of chloroplasts with intact membranes which were capable of high rates of photosynthesis without addition of cofactors or enzymes. They considered these preparations to be more useful than reconstituted systems for the study of metabolic control and secondary biosynthetic pathways following CO_2 reduction.

As well as internal factors which can influence the rates of photosynthesis, external factors in the immediate environment of the chloroplast also have an effect. Ellyard and Gibbs (16) have shown that spinach chloroplasts fixing CO_2 under O_2 show a longer lag than under N_2, but finally

attain a similiar rate of CO_2 incorporation. This lag can be reduced by fructose-1,6-diphosphate and by ribose-5-phosphate. The inhibition is reversible, the lag being shortened by switching to N_2. The rapidity of this recovery is proportional to the bicarbonate concentration. Chloroplasts fixing CO_2 under O_2 have lower levels of ribulose-1,5-diphosphate and triose phosphate. By contrast these chloroplasts accumulate greater amounts of pentose monophosphates and glycollic acid in O_2 than in N_2. Again, a dependence on the bicarbonate concentration exists. They found that high O_2 concentrations and low bicarbonate concentrations resulted in up to 90% of the label appearing in glycollate. Chloroplasts were also shown to have different pH optima for photosynthesis under N_2 and O_2.

In order for the reactions of the chloroplast to proceed it is first necessary to convert the radiant energy of light to that of chemical energy. This is accomplished by a system of pigments. A review by McLeod and French (36) showed that the concentration of chlorophyll in leaves made it apparent that the excitation of any one chlorophyll molecule by an average flux of photons would require about 20 to 30 minutes for the initial reduction of CO_2 by that same molecule. Since it is possible to obtain initial rates without induction periods, some means of energy transfer from molecule to molecule must exist. The primary absorber may be some non chlorophyll molecule - the phycobilins or

the carotenoids. For example, the action spectra of Nitzchia photosynthesis, shows that the amount of light absorbed in the blue region by chlorophyll is insufficient to account for all the photosynthesis obtained. By subtracting the fraction of photosynthetic yield contributed by chlorophyll from that actually found, a curve of the absorption spectrum of the contributing pigment was obtained. These curves were found to fit one or more of the carotenoids present. Since carotenoids are the only plant pigments present in amounts sufficient to account for the amounts of light absorbed, some of the carotenoid family must be active in photosynthesis. Since no plants are known where photosynthesis proceeds in the absence of chlorophyll, even though carotenoids are present, it can be infered that there is an energy transfer relationship between the two pigments.

The pigment families absorb in the visible region of the electromagnetic spectrum, and within this region the blue and the red are the major absorbing bands. In photosynthesis under normal light conditions, all the wavebands contribute to the final product simultaneously. In 1957, Emerson (17) performed some studies on the effect of isolated light bands from the longwave red region supplemented with light from a different region. He found that the yield in Chlorella from photosynthesis centered on the 644 mu band was not increased by supplementary red illumination

from another region. Wavelengths from 644 mu to 436 mu with intensities capable of increasing photosynthesis rates 10 times that of respiration rates were used. The yield of photosynthesis from a band of light longer in wavelength than 690 mu could be increased by supplementary light of shorter wavelengths. The wavelengths of supplementary light found to be most effective were 578 mu and 644 mu. The least effective was the waveband centered on 436 mu. Emerson stated that a comparison of these results with the absorption spectra of chlorophylls a and b showed that there was a possibility of interpreting the difference in effectiveness of the different wavelengths as evidence that chlorophyll b is the sensitizer for the action of supplementary light.

For plants containing chlorophyll a and b and the conventional carotenoids, photosynthetic effectiveness follows the combined absorption curves of the chlorophylls (24). It has been estimated that 40% to 50% of the light absorbed by the carotenoids is available for photosynthesis. The exact extent to which these pigments are active remains uncertain. The common occurrence of carotenoids in the photosynthetic apparatus of plants suggests that they may play an important role other than that of trapping light for photosynthesis - possibly protection of the chlorophylls against damage by light (30). Krotkov (32) advanced two tentative hypotheses to explain the influence of the wavelength of incident light on the pathways of absorbed carbon

in photosynthesis. According to the first, one of the enzymes involved in the early stages of photosynthesis has a pigment as its prosthetic group which is activated by some particular wavelength. Depending on the form in which this enzyme is present, it directs the flow of early intermediates of CO_2 assimilation along different paths. According to the second hypothesis, the light reaction in photosynthesis consists of two photochemical acts. Each act is brought about by a different wavelength absorbed by a different pigment, with the subsequent appearance of different reductants and photosynthetic products. The spectral composition of the incident light determines the relative importance of the two photochemical acts and this in turn affects the path of carbon in photosynthesis.

Borthwick and Hendricks (11, 27) suggested that phytochrome pigments, present in two forms which are reversibly interconverted by low intensity radiation of the red and infrared bands, could control a reaction of the prosthetic group of some strategically placed enzyme. Depending on the form in which the phytochrome is present after irradiation the flow of metabolites may be directed either along one course or another. It may be that one of the known pigments or some as yet unisolated pigment of the photosynthetic mechanism behaves in a similar manner.

Effects of Light Quality on Photosynthetic Products

Within the last few years, several workers have summ-

arized available data to indicate that light quality affects the distribution of absorbed carbon among the various products of photosynthesis (3, 31, 38, 39, 45, 51). Voskresenskaya (49) has shown that in leaf discs from several plants illuminated with blue light (450 - 580 mu) protein synthesis is increased while the formation of carbohydrates is decreased as compared with red light (580 - 700 mu) at an equal number of quanta. Cayle and Emerson (12) illuminated Chlorella for 30 seconds with equal intensities of blue (436 mu) and red (644 mu) and found that red light radioactivity of the amino acids was 74% as compared with the blue. Degradation studies of glycine revealed that the portion of ^{14}C in carboxyl and alpha carbons was 42:57 in red light but 22:78 in blue light. Hauschild, Nelson, and Krotkov (22, 23) illuminated Chlorella, Blue Green and Green algae and photosynthetic bacteria with red, red and 4% blue and blue light . After five minutes of photosynthesis, the incorporation of ^{14}C into aspartic acid was higher in the red and 4% blue than red light alone. At the same time the total radioactivity in glycine, serine and glycollic acid decreased. After 30 minutes of photosynthesis, blue light supplemented with red and blue light alone had an increased effect on the total incorporation of ^{14}C in the amino acid and organic acid fraction as compared with red light. These results suggest an effect of light quality not only on the relative amounts of compounds produced, but also on the pathways by which they are formed.

Champigny (13) found that the amount of carbon fixed in Bryophyllum leaves affected the relative distribution among products. For small amounts of $^{14}CO_2$, more label appeared in the amino acids than carbohydrates. This would seem to indicate a dependence on the CO_2 concentration as well as the light quality for the pathways by which compounds are formed.

Protein Synthesis

It is well known that most of the higher plants do not synthesize chlorophyll when growth occurs in the dark. They become etiolated and the cells of their leaves contain yellowish plastids known as leucoplasts. Etiolated leaves are known to be poor in chloroplastic proteins, while at the same time cytoplasmic proteins appear normal. During the greening of etiolated leaves on exposure to light, synthesis of chloroplastic proteins occurrs and the leucoplasts are transformed into chloroplasts. A comparison between plastids from etiolated and green leaves with the electron microscope (14) shows a radically different structure in these two types of particles. The leucoplasts do not contain grana, but show a finely granular structure. They are smaller than chloroplasts. The light induced transformation of the leucoplasts to chloroplasts is accompanied by a significant net synthesis of protein at the expense of free amino compounds which are in considerable reserve in the etiolated leaves.

Chloroplast synthesis has been shown to be reduced by

various culture conditions and is preferentially inhibited by inhibitors of protein synthesis (15). Exposure of cultures of Euglena gracilis to elevated temperatures, UV radiation or streptomycin under conditions having little effect on cell proliferation results in irreversible bleaching of the cells. These cells have apparantly lost the system responsible for the self replication of the chloroplasts. Microbeam irradiation of the nucleus or the cytoplasm alone indicates that the irreversible bleaching of the cells occurrs only when the plastids are exposed. This may be correlated with the observation that bleached cells no longer contain the chloroplast associated DNA and it implies that the DNA of the plastids did not originate from the nucleus. Recent findings (15) that chloroplasts contain ribosomes which, by the criteria of size, RNA content and biochemical properties are sharply different from cytoplasmic ribosomes, has lent additional support to the concept that chloroplasts possess an independent protein synthesizing system. Eisenstadt (15) obtained results indicating that the isolated chloroplasts of Euglena gracilis are capable of incorporating amino acids into proteins and that this synthesis is different from that found in the cytoplasm.

The incorporation of labeled amino acids into protein fractions of chloroplasts has been reported by Bamji and Jagendorf (6) as well. Chloroplasts of Phaseolus vulgaris after incorporation of leucine-^{14}C for one hour were assayed

by Margulies and Parenti (35). They found half the protein formed in a soluble fraction, and the other half in an insoluble fraction. They concluded that the system under study was synthesizing both soluble and lamellar chloroplast proteins. Possibly two separate incorporation sites were involved since the soluble fraction did not act as a precursor for the insoluble and vice-versa.

The destruction of chloroplasts after $^{14}CO_2$ photosynthesis in leaves of Nicotiana rustica yielded two fractions, the soluble and the lamellar proteins. The lamellar fractions specific activity exceeded that of the soluble portion (10). Heber (25) found that in chloroplasts undergoing $^{14}CO_2$ photosynthesis, radioactive compounds were incorporated into protein as soon as photosynthesis was initiated, but that cytoplasmic incorporation showed a lag. An early indication that the free amino acids from a free amino acid pool did not serve as precursors in protein synthesis was provided by Gregory and Sen (20) and based on their work with barley leaves. Thompson and Steward (45) found no proportionality between different amino acids in the free and bound state in potato tubers. They concluded that little or no significance was to be attached to the amino acids which occurr free as direct intermediates of protein synthesis. Steward, Bidwell and Yemm (44) have reported further evidence for the assumption that carbohydrates may be the chief source of carbon for protein synthesis from work on carrot tissue cultures. They

noted that the wide variation in the specific activity of the free amino acids was not reflected in the labeling of protein amino acids. However, it has been shown by Smith, Bassham and Kirk (42), and Bassham et al. (7) that there may be several pools of amino acids in plants. Hellebust and Bidwell (26) with their work on protein synthesis in snap dragon and wheat leaves have calculated the proportion of carbon entering some protein amino acids which came either from soluble amino acid pools or by a direct route from photosynthate, bypassing the soluble pools. They found more than half of the carbon entering protein bound serine and glycine to be derived from newly assimilated CO_2, while protein glutamic acid, aspartic acid and alanine derived more of their carbon from the soluble amino acid pools.

Assimilation of Ammonia

Voskresenskaya (48) reported that synthesis of protein in illuminated leaves occurred only in the presence of CO_2 and the protein synthesis in light increased when $(^{15}NH_4)_2SO_4$ was allowed to infiltrate the leaves. In an investigation of the utilization of inorganic nitrogen by Chlorella, Reisner et al. (40) measured the changes in the quantities of uncombined amino acids which occurred after adding nitrate or ammonia to nitrogen starved cells. The addition of ammonia produced more rapid and more striking changes in amounts of amino acids than adding nitrate. After feeding ^{15}N labeled

ammonia to plants, the relatively high ^{15}N content of glutamic acid had been noted by several workers (34, 47, 54). After feeding barley ^{15}N ammonia for short periods of time, glutamic acid had the highest ^{15}N content.

Bassham and Kirk (8) performed dual tracer experiments using ^{15}N and ^{14}C with Chlorella under steady state photosynthesis. Their results indicate that the reductive amination to form glutamic acid is the primary route for the incorporation of ^{15}N during photosynthesis of the amino acids. The relation between the time of maximum labeling of glutamic acid and the maximum rate of labeling of the other amino acids studied clearly indicates that the amino groups in the other acids could all have arisen by transamination of their respective keto acids by glutamic acid. They believe there is no reason to think that amino acids other than glutamic acid incorporate ammonia by a direct reductive amination. The rate of labeling of the amino group of glutamic acid when estimated was shown to account for most of the NH_4^+ taken up.

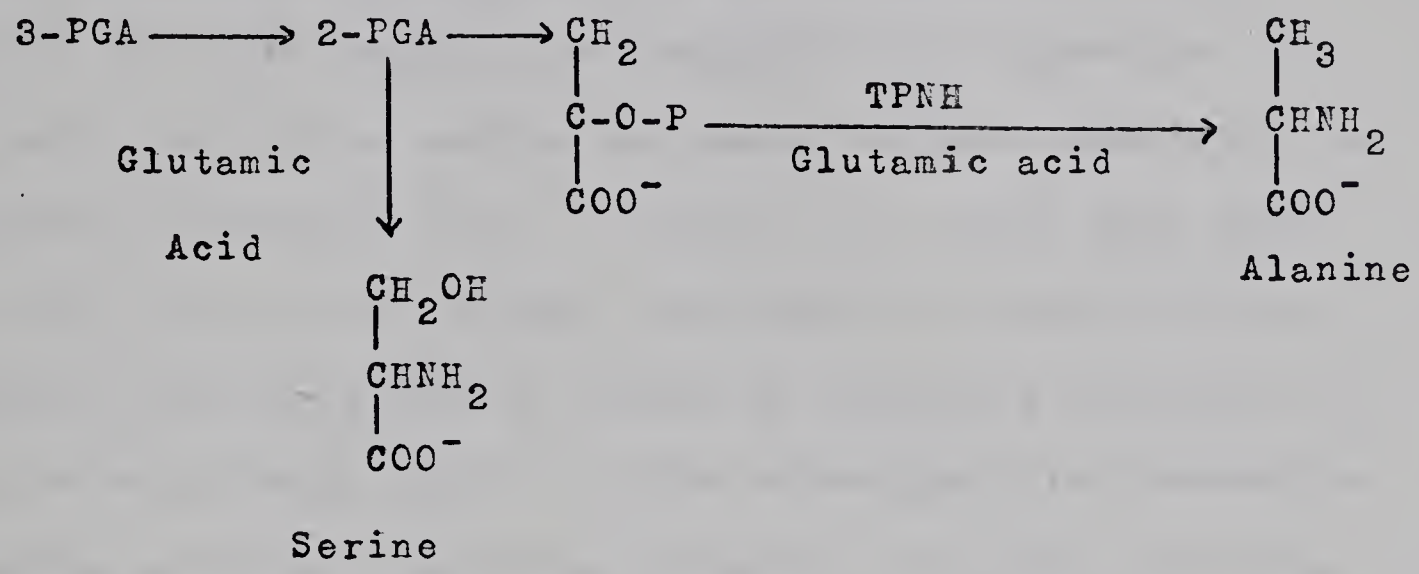
Baker and Thompson (5) found with Chlorella that the ^{15}N content of glutamic acid was considerably higher than that of alanine after the administration of labeled ammonia. On the basis of their work they concluded that the alanine nitrogen must have been derived from glutamic acid.

Pathways

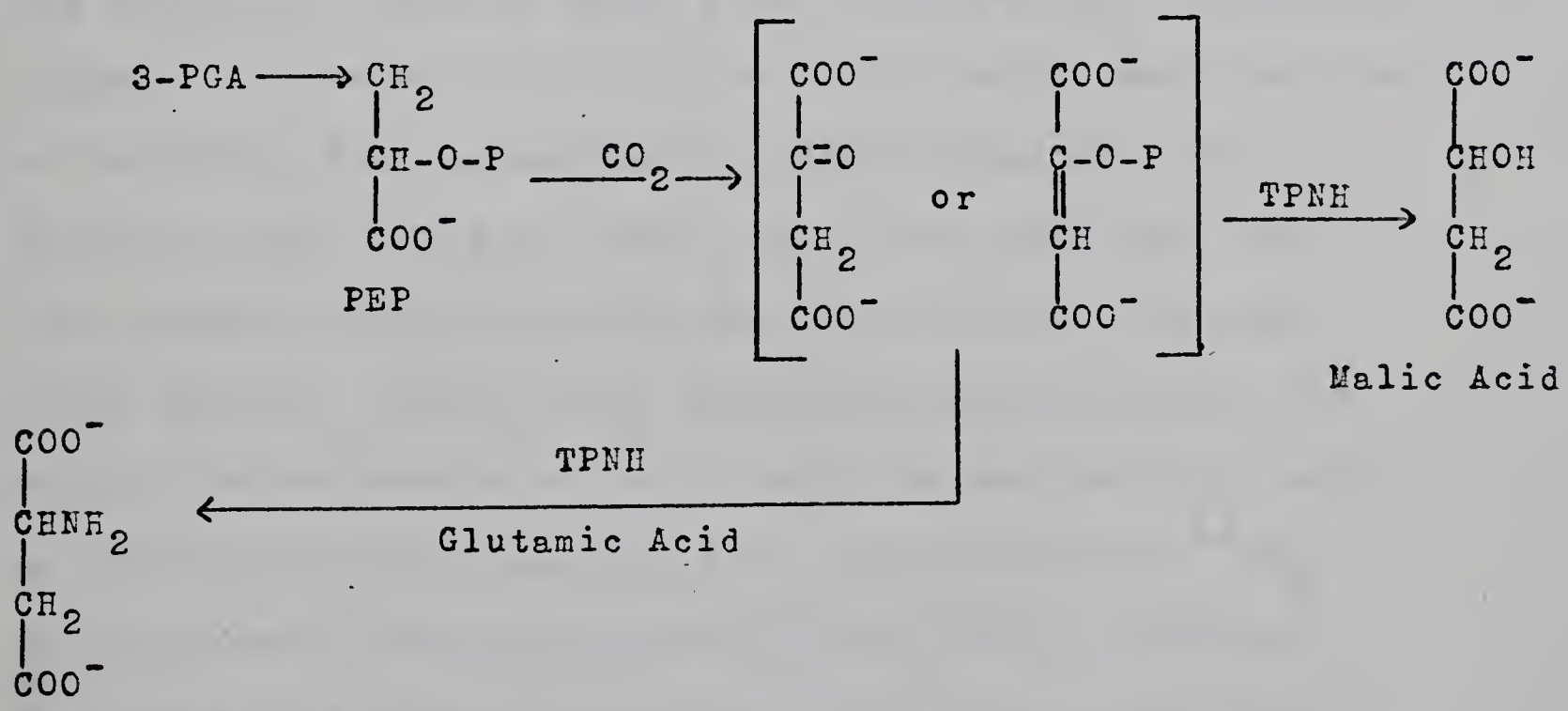
The ^{14}C distribution in molecules of phosphoglyceric acid, glycine and serine and alanine upon exposure of Chlor-

ella in the presence of ^{14}C to light was studied by Zak and Nichiporovich (55). They demonstrated that alanine and serine were formed as a result of the further conversion of the first stable product of photosynthesis, phosphoglyceric acid. They found that the distribution of ^{14}C in the glycine molecule did not confirm it as being the direct precursor of serine. It is more probable that serine is formed by the pathway of nonreductive amination of 2-phosphoglyceric acid in the first few seconds of photosynthesis when the glycollate pathway has not yet reached any degree of significance. Smith, Bassham and Kirk (42) performed experiments on the kinetics of the rates of appearance of ^{14}C in individual compounds formed by Chlorella during steady state photosynthesis. From their experiments they concluded that synthesis and utilization of alanine, serine, aspartic acid, glutamic acid and several other amino acids are most active within the chloroplast during photosynthesis, and that these amino acids are formed rather directly from the intermediates of the carbon reduction cycle. If the principle site of amino acid synthesis is the chloroplast, it can be expected that the environment within the chloroplast would influence the biochemical pathways of amino acid synthesis. This environment varies with the supply of TPNH, ATP and carbon incorporated into PGA, PEP and various sugar phosphates. They found that alanine accounted for about half of all synthesis from carbon of the amino acids studied. It seems likely that alanine is

synthesized from intermediates of the carbon reduction cycle directly by reductive amination. If the transamination and reductive amination of pyruvic acid are eliminated as routes to alanine synthesis, the following scheme seems to be the most probable.



Serine may be formed by a similiar but non reductive amination of 2-PGA. The rapid labeling of aspartic acid which they found was considered as evidence for the carboxylation of PEP.



The photosynthetic formation of glycine appears to depend on

the prior formation of glycollic acid (9). Presumably glycollic acid is oxidized to glyoxylic acid which is transaminated to give glycine. Smith, Bassham and Kirk (42) suggested that glutamic acid and glutamine are formed in the chloroplast independently of a common precursor which is in turn formed by condensation and reduction of two carbon and three carbon compounds derived directly from the carbon reduction cycle. Bassham (9) stated that glutamic acid which is of primary importance in photosynthesis of amino acids is probably formed by reductive amination of alpha keto glutaric acid. In the chloroplast the reductive amination utilizes electrons formed by the light reactions of photosynthesis. The alpha keto glutaric acid is formed from citric and oxalosuccinic acids via the krebs cycle. The reservoir sizes of these acids along the photosynthetic pathway to glutamic acid must be rather small, and the rate of labeling of these compounds appears less than that of glutamic acid. This may have led to the suggestion (42) that glutamic acid may arise photosynthetically by some other pathway. Hiller (28) used fluoroacetate, which inhibits the conversion of citric acid to oxalosuccinic acid, on photosynthesizing Chlorella in the presence of $^{14}CO_2$. Glutamic acid labeling stopped and the rate of labeling of citric acid became as great as that of glutamic acid in the uninhibited algae. Bassham (9) suggested that there is a small but rapidly turning over pool of citric acid in

the chloroplast. This small pool must be quickly saturated with ^{14}C during photosynthesis with $^{14}CO_2$. Thus ^{14}C is passed through this pool and on to glutamic acid.

MATERIALS AND METHODS

Plant Material

Leaf material from a number of varieties of plants including spinach (Spinacia oleracea), both store bought and freshly grown, beans (Phaseolus vulgaris) and soybean (Soya max), were used in initital studies. Kinghorn wax beans (Phaseolus vulgaris) were eventually chosen for the study because of their uniform and rapid rate of growth and the relative ease of chloroplast preparation.

Every/day 25g of seed were sown in 12" X 12" trays filled with and covered by a one inch layer of vermiculite. The media was soaked with tap water and the trays were placed in a growth chamber maintained at 15° C, 65% relative humidity and under 1700 ft-c of continous illumination. Every two days 500 ml of tap water were added to each tray. The leaves were harvested when the seedlings were 12 days old. Prior to harvest for chloroplast preparation the seedlings were placed in darkness for 12 hours. This pretreatment was found necessary to enhance the difference between the light and dark controls.

Isolation of the Chloroplasts

Forty grams of leaf material in 60 ml of 0.33M sucrose phosphate buffer (details given in appendix) were ground gently in a mortar and pestle with sand at 4° C. The pulp was squeezed in 4 layers of cheeze cloth to separate the

crude homogenate. The homogenate was centrifuged in an International Equipment Company Model BD-2 centrifuge at 100g (1500 rpm) to remove sand and cell debris for one minute. The supernatent was then centrifuged at 1000g (4000 rpm) for 15 minutes to yield the crude chloroplast pellet.

Purification of the Chloroplasts

The crude chloroplast pellet was purified on a discontinous sucrose density gradient (see appendix). The discontinous gradient was prepared as follows: 6 ml of 1.5M sucrose phosphate buffer were layered with a ten ml syringe on 6 ml of 2.0M sucrose phosphate buffer. On top of this were layered 6 ml of 1.0M sucrose phosphate buffer. The crude chloroplast pellet was suspended in 10 ml of 0.33M sucrose phosphate buffer and 5 ml of this suspension were layered on the 1.0M sucrose phosphate band. The gradients were then subjected to centrifugation at 25,000g (17,000 rpm) for twenty minutes. After sedimentation, the chloroplast layer was retained at the interface between the 1.0M and 1.5M sucrose bands. The liquid above this layer was removed with a syringe and discarded. The chloroplast layer was then removed in a similar manner. An equal volume of sucrose free phosphate buffer was added to the removed chloroplast layer and the suspension centrifuged at 3000g (6000 rpm) for 15 minutes. The pellet obtained from this centrifugation was considered as the purified chloroplast

pellet.

Microscope Studies

Slides of the purified chloroplasts were prepared by placing a drop of the final suspension on a microscope slide and covering it with a cover slip. The slides were examined immediately under a microscope for intactness and purity of the chloroplast suspension and photographs taken.

Soluble and Protein Amino Acids

The purified chloroplast pellet was ground in a mortar and pestle with 10 ml of cold 10% trichloroacetic acid (TCA). The suspension was rinsed into a centrifuge tube and centrifuged at 25,000g for twenty minutes. The pellet was washed twice with 10% TCA and the washings and origional supernatant combined to yield the fraction designated as soluble amino acids. Amino acid analysis will be described later.

Isolation of Chloroplast Pigments

The purified chloroplast pellet was suspended in 30 ml of an 8:2 acetone water mixture and ground in a mortar and pestle. The liquid was filtered through Whatman #42 filter paper into an evaporating flask and then flash evaporated to dryness at a temperature below 40^{o} C. The pigment residue was then taken up in a mixture of 1:1 benzene petroleum ether and filtered through a pad of anhydrous sodium sulfate. The filtrate was finally evaprotaed to a volume of 10 ml.

Determination of Total Chlorophyll

The method of Mackinney (33) was used. A 0.5 ml aliquot of the purified chloroplast suspension was transfered to a 25 ml volumetric flask containing 4.5 ml of distilled water. This was made up to volume with acetone, shaken and filtered through Whatman #42 filter paper. The absorbance of the filtrate was read at 652 mu on a Bausch and Lomb Spectronic 20. The approximate chlorophyll concentration was calculated from the following formula.

$C = A_{652} \times 50/34.5$ = mg chlorophyll/ml (33)

Separation of the Individual Pigments

Chlorophylls a and b and the carotenoids were separated from one another by a modification of the method of Smith and Benitez (43). Commercial icing sugar was dried in an oven at 110° C overnight. The solvent mixture used, 12:12:1 benzene petroleum ether acetone, was stored over anhydrous sodium sulfate to remove traces of water. It was necessary to add acetone to make the solvent sufficiently polar to prevent chlorophyll b from being too tightly held to the column, yet not polar enough to completely destroy separation between chlorophyll a and the carotenoids. A 0.9 X 60 cm column was packed with the dried icing sugar in the following manner. The column was filled to 20 cm with the solvent mixture. A slurry of the icing sugar and solvent mixture was stirred to remove all air and then poured into the column. Pressure was applied with a constant volume delivery

pump to settle the packing. Consecutive slurries were thus introduced till the column was packed to a height of 50 cm. After addition of the last slurry, solvent mixture was pumped through the column for four hours to ensure uniformity throughout the packing. An aliquot of 1.0 ml of the concentrated pigment extract in 1:1 benzene petroleum ether was placed on the column and forced into the packing with nitrogen pressure. The sample was then pumped with a constant volume delivery pump at the rate of 80 ml/hour. Two ml fractions of the eluent were collected with a fraction collector. This separation was carried out in a dark room equipped with a green safelight. The separate fractions were read at two wavelengths, 652 mu for the chlorophylls and 672 mu for the carotenoids to obtain the elution curve of the separated pigments. From this curve the fractions were selected so as to obtain the isolated pigments in purest form. A full spectrum of these individual purified pigments from 350 mu to 750 mu was taken on a Perkin Elmer 202 recording spectrophotometer.

Reaction Mixture

The standard reaction mixture substrate was made up by dissolving 0.0258 grams of anhydrous sodium carbonate in 50 ml of 0.33M sucrose phosphate buffer. To this was added 1 mc of $Na_2{}^{14}CO_3$. For certain experiments $(NH_4)_2SO_4$ was added to the standard reaction mixture to give a final concentration of 2 u moles/ml. This mixture was stored in

a frozen state and was thawed each time just prior to use. The purified chloroplast pellet referred to earlier was suspended in 6.0 ml of 0.33M sucrose phosphate buffer and 0.5 ml of this suspension along with 0.5 ml of substrate were added to each reaction vessel. The reaction was started by turning on the lights and starting the shaker. After one hour the reaction was terminated by the addition of 0.5 ml 20% TCA.

Separation of Soluble and Non-Soluble Portions

Following the addition of the TCA the reaction mixture was drawn off into centrifuge tubes and the cells rinsed with distilled water, the washings being combined in the centrifuge tubes. The mixture was then centrifuged at 25,000g (17,000 rpm) for twenty minutes. The supernatant was poured into a beaker and the insoluble portion or pellet was rinsed twice with distilled water and recentrifuged. The washings were combined with the supernatant in the beaker.

Isolation of Free Amino Acids

A column of 1 X 5 cm of Dowex 50-X8 (200 to 400 mesh) resin in the hydrogen form was prepared in the following manner. Fifty ml of 2N HCl were allowed to drain through the resin. This was followed with distilled water till the eluent attained a pH of 5.5 to 6.0.

The pH of the combined washings and supernatant was adjusted with HCl or NH_4OH to a pH of 2 or less and was rinsed onto the column. The resin was washed with 100 ml

of distilled water to remove all the buffer salts, sugars, organic acids and other soluble products from the amino acids. Recovery experiments with known quantities of aspartic acid and arginine in the concentration range of the unknown samples yielded a consistent recovery of 98% of the added acids. The amino acids, after washing of the resin, were eluted with 50 ml of 2N NH_4OH into evaporating flasks. The material was then flash evaporated to dryness at a temperature below 40° C. Twenty ml of 2N HCl was added to the dried flasks and the contents hydrolyzed for three hours in an autoclave at 16 lbs pressure. This was found to be sufficient for hydrolyzing any peptides present. After autoclaving the flasks were removed and the contents again flash evaporated to dryness below 40° C. Samples were prepared by adding 10 ml of sample diluting buffer (see appendix) to the dried contents of the evaporation flasks. The liquid was transfered to test tubes, stoppered with parafilm wrapped corks and stored in the cold room till analyzed.

Isolation of Protein Amino Acids

To the pellet sedimented by centrifugation were added 20 ml of constant boiling HCl. The flask was covered with a beaker and placed in an autoclave. The contents were hydrolyzed for 6 hours at a pressure of 16 lbs. Hydrolysis of plastid material for different lengths of time were carried out to determine the time required for complete hydrolysis. After hydrolysis for 4 hours a large peptide peak

occurred when a sample was analyzed. Also the amounts of other acids varied significantly between duplicate samples. After hydrolysis for 6 hours, the peptide peak had disappeared and there was no longer any significant difference between duplicate samples. Hydrolysis for 8 and 10 hours produced no change in the amounts of acids between samples. After hydrolysis for 16 hours there was a slight constant decrease in the amounts of acid present. It was assumed that destruction of the acids had begun to occurr at this point. The 6 hour hydrolysis time for plastid material was therefore selected. After hydrolysis, the contents of the flasks were filtered through sintered glass into evaporation flasks. The contents were then flash evaporated to dryness at 50° C. Samples were prepared by adding 10 ml of sample diluting buffer to the flask contents. The liquid was transfered to test tubes, stoppered with parafilm wrapped corks and stored in the cold room till analyzed.

Chromatographic Separation and Analysis of Samples

The chromatographic separation and analysis of the labeled amino acid samples were carried out on a Spinco/Beckman amino acid analyzer and a Nuclear Chicago liquid scintillation counter. A solid scintillator flow cell was inserted into the counter, the inlet comming directly from the outlet of the chromatographic tube on the amino acid analyzer and the outlet going back to the reaction system of the analyzer. The counter was set to print out the accumulated

counts per minute. The amino acid analyzer used columns packed to a height of 50 cm and 10 cm with Beckman type AA-15 and AA-27 resins respectively. The columns were operated at 55° C with a flow rate of 68 ml per hour. Sodium cyanide in the eluting buffer (see appendix) at a concentration of 0.00004M was used as the catalyst for color development of the amino acids. Ninhydrin reagent (see appendix) was used for the color development and was pumped at a flow rate of 34 ml per hour. Samples consisting of 1 or 2 ml were applied to the top of the resin and forced into the resin with nitrogen pressure. The run was commenced by switching on the eluting buffer pump and the counter.

Construction of Light Filters

The specifications developed by Zalik and Miller (56) and shown in table I were followed in the construction of a light filter box consisting of ten different compartments. The incandescent lamps used as the light source were 6 volt, 15 to 20 watt single and double filament General Electric automotive lamps. The lamps were individually connected to a rehostat to enable the adjustment of light intensity. The divisions between the different compartments, the top and the bottom of the box (see fig. 1) were constructed of black perspex to eliminate stray light. Water thermostated at 25° C was circulated around the test tubes in the sample compartments. The exit flow from the sample compartments

TABLE I

Composition and Transmittance Data on Filters

Wavelength mu 50% Transm.	Peak	Color Designation	Plastic	$CuSO_4$ g/l
Dark	-----	-----	----	----
393 - 463	425	Blue	705* & Clear	50.0
436 - 492	465	Blue	2085 & 2082	150.0
460 - 532	493	Green	2082 & 2082	75.0
500 - 572	532	Green	2154 & 2208	20.0
540 - 605	560	Yellow	2451 & Clear	10.0
580 - 655	600	Orange	2451 & 2085	3.0
630 - 675	660	Red	2444 & Clear	9.0
720 - 900	800	Far Red	Clear & FRF 700**	0.15
Clear	Clear	-----	Clear & Clear	0.15

* Manufactured By Imperial Chemical Industries Limited.

** Manufactured By Westlake Plastics Company.

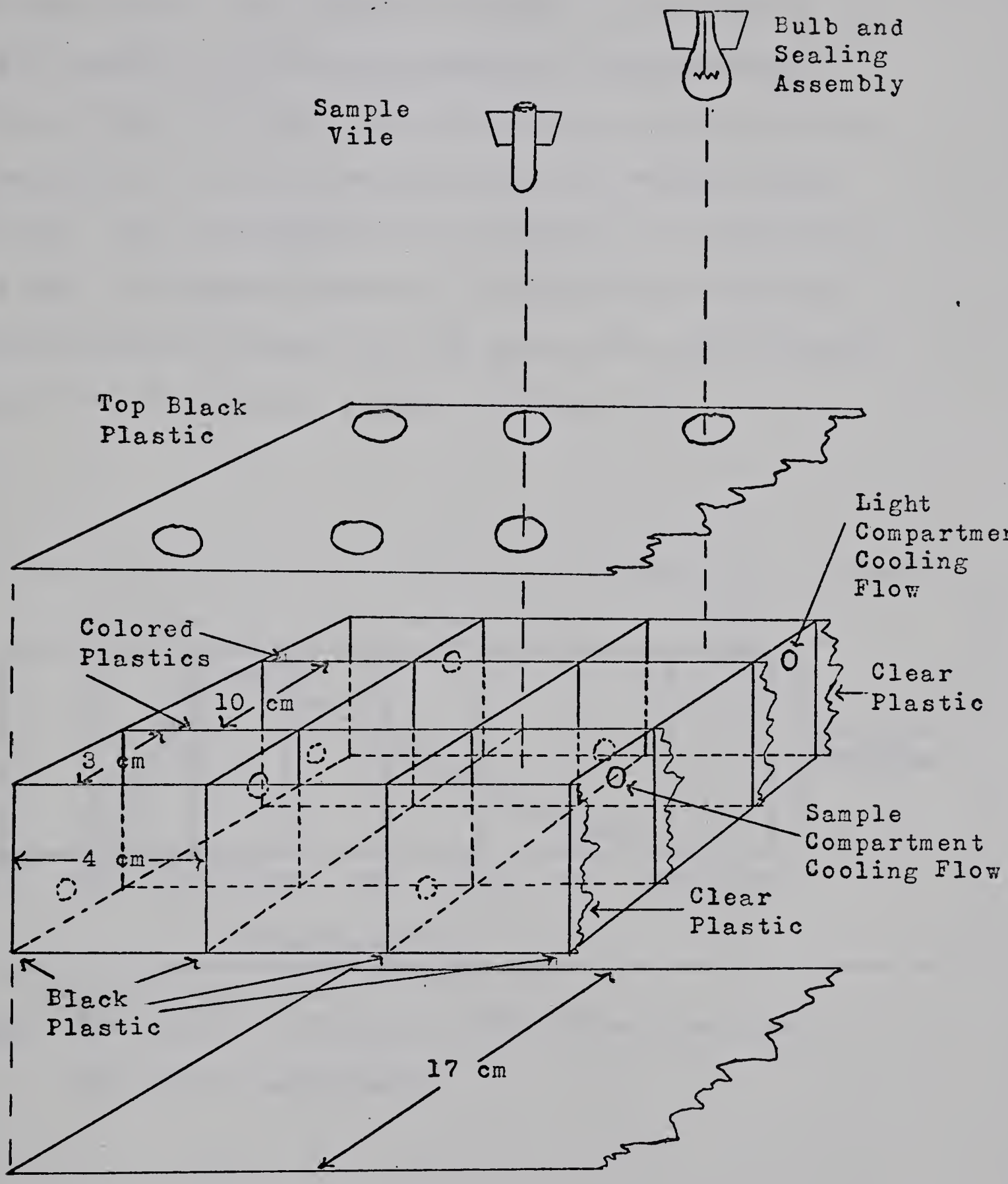
Other plastics were products of Rohm and Hass Company.

was directed through the lamp compartments to cool the lamps. The lamps were fitted in rubber stopper bases with wires soldered directly to the lamp base. The individual lamps were then placed into the appropriate lamp compartments with the rubber stoppers sealing the entry hole on top. The exit flow from the lamp compartments was passed through two heat exchanger coils surrounded by cold flowing tap water. The exit flow from the heat exchanger was passed back into the thermostated bath. This system was found adequate in preventing the lamps from overheating and in maintaining a constant temperature of 25° C in the sample compartments. The entire assembly was firmly attached to a rotary action shaker.

Calibration of the Light Filters

A phototube of type E connected to a Photovolt Electronic Photometer Model 501-M was used for the purpose of calibration and adjustment of the energies of the different compartments. To allow for the variation in the response of the phototube to the different wavelengths (see fig.2) and in order to set each cell for the same amount of energy, a series of correction factors (see table II) were calculated from the graph corresponding to the wavelength of peak transmission for each filter. Applying these correction factors, the cells were all set to the same energy that could be attained as a maximum in the blue filter. The window of the phototube was set against the clear plastic of each

Fig. 1 Details of light filter construction showing compartment arrangements, light assemblies and sample tubes.


Bulb and
Sealing
Assembly
Sample
Vile
Top Black
Plastic
Light
Compartment
Cooling
Flow
Colored
Plastics
10 cm
3 cm
4 cm
Clear
Plastic
Sample
Compartment
Cooling Flow
Clear
Plastic
Black
Plastic
17 cm
Bottom
Black Plastic

compartment and the rehostat adjusted till the desired meter reading was attained. The phototube E was calibrated with white light against an Epply pyroheliometer of the 180° weather bureau type hooked to a nanovoltmeter Astrodata Model TDA-121. The calibrated constant for the pyroheliometer at low intensity radiation was 306 ergs/ cm^2/ sec. This corresponded to a reading of 1 microvolt. Using this calibrated constant, the phototube calibrated at 68.74 ergs/cm^2/ second for one corrected scale division on the 0 to 100 scale at a range setting of 0.

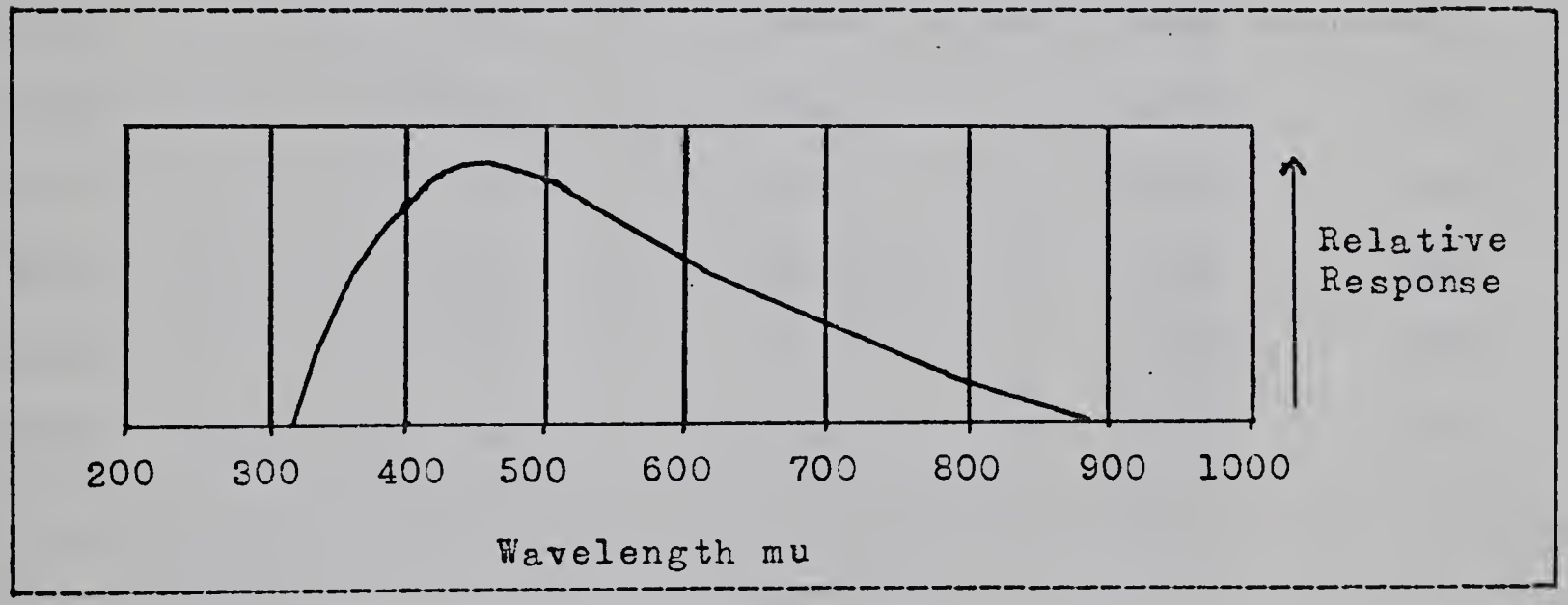


Fig. 2 The relative response of Phototube E to light of various wavelengths.

TABLE II

Lamp Wattage and Calculated Energies

Peak Wavelength mu	Lamp Wattage	No. of Div. on Photometer	Correction Factor	Energy Calcu. Erg/cm^2/sec
Dark	0	----	-----	------
Clear	5	30.0	1.00	2062
425	20	29.0	1.04	2090
465	20	29.5	1.00	2028
493	20	28.0	1.05	2021
532	15	26.0	1.17	2097
560	15	22.5	1.35	2090
600	20	18.5	1.59	2021
660	20	15.5	1.86	1980
800	5	5.0	6.11	2097

RESULTS

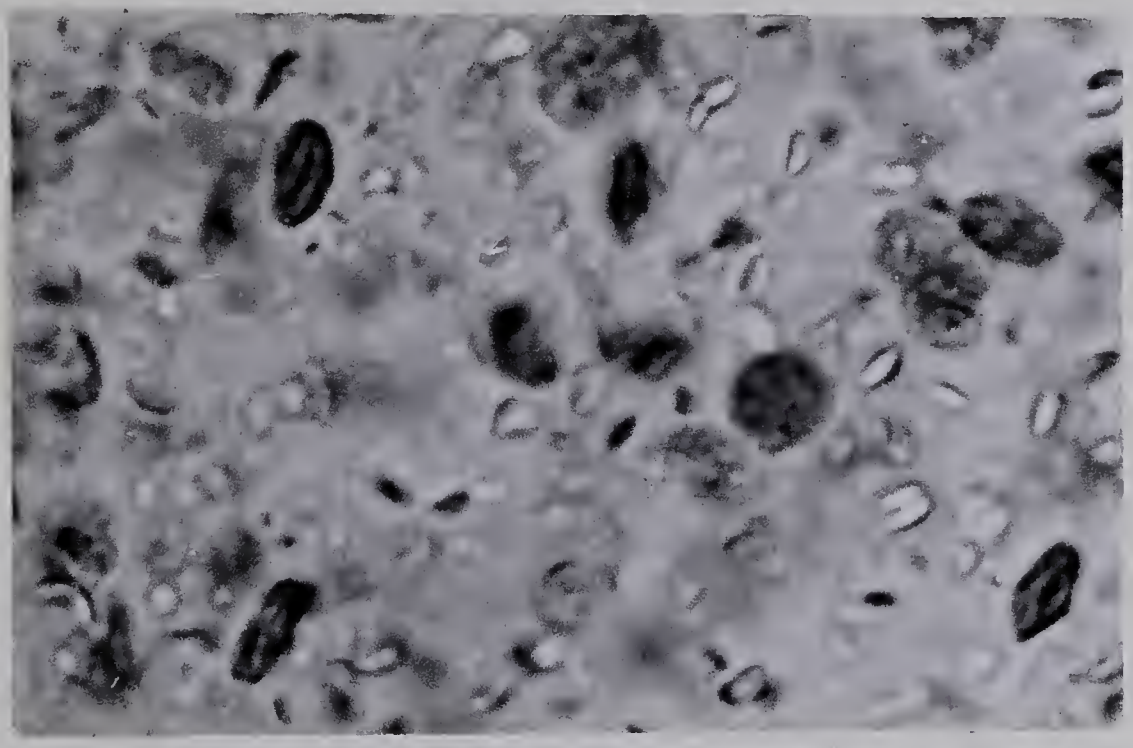
Chloroplasts

The results showing the intactness and purity of the chloroplast suspensions isolated are presented in fig. 3.

Soluble and Protein Amino Acids

The results for the soluble and protein amino acids isolated from the purified chloroplasts of Kinghorn wax beans are presented in table III.

Figure 3



Light micrograph of isolated Kinghorn wax bean chloroplasts purified on a sucrose density gradient. The small particles are intact chloroplasts out of focus. Focusing different layers within a field showed no chloroplast fragments or other organelles at this magnification. Magnification X 6300.

TABLE III

Soluble and Protein Amino Acids of Purified Chloroplasts From Kinghorn Wax Beans

Amino Acid	u moles Soluble Amino Acid	u moles Protein Amino Acid
Lysine	0.125	5.92
Histidine	0.115	1.73
Arginine	0.065	2.98
Aspartic Acid	0.220	17.46
Threonine	0.120	16.91
Serine	0.290	21.56
Glutamic Acid	0.170	15.26
Proline	0.140	10.92
Glycine	0.205	16.90
Alanine	0.190	13.24
Isoleucine	0.055	6.90
Leucine	0.160	14.39
Tyrosine	0.045	10.41
Phenylalanine	0.065	8.79
Valine	0.120	0.81

The results are averages of two determinations each based on the chloroplasts isolated from 40g of the fresh leaf material.

Chloroplast Pigments

Fig. 4 presents the elution curve of the pigments separated on the sucrose column. The shaded areas represent the fractions collected for obtaining the absorption curves of the individual pigments shown in fig. 5. The two major absorption peaks for the total carotenoids occurred at 408 mu and at 672 mu. For chlorophyll a the major peaks were 430 mu and 660 mu. Chlorophyll b gave major absorption peaks at 456 mu and 640 mu.

$^{14}CO_2$ Feeding Experiments With and Without Ammonia

When purified chloroplasts were incubated with or without ammonia in the reaction mixture the amount of label incorporated into soluble amino acids was greatest in the blue and red regions of the spectrum (tables IV and V, fig. 6). The total amount of label incorporated into the soluble amino acid fraction was higher than that incorporated into protein whether ammonia was present or absent. It can be seen from fig. 6 that in the blue region there was a 200% to 300% increase in labeling over the dark control in the absence of ammonia. The maximum increase in the red was about 50%. For the experiments with ammonia present, the blue region still incorporated approximately 200% as much label as the dark control, but the red band now incorporated 150% as much label as the dark control. The total incorporation of label into the soluble amino acid fraction was ten times more in the presence of ammonia

Fig. 4 The elution curve from a sucrose column of the pigments isolated from purified Kinghorn wax bean chloroplasts. The shaded block portions represent the fractions combined for the absorption spectra of the individual pigment.

Fig. 6 The incorporation of ^{14}C by purified wax bean chloroplasts into soluble and protein amino acids with and without ammonia in the reaction mixture during incubation.

Figure 4

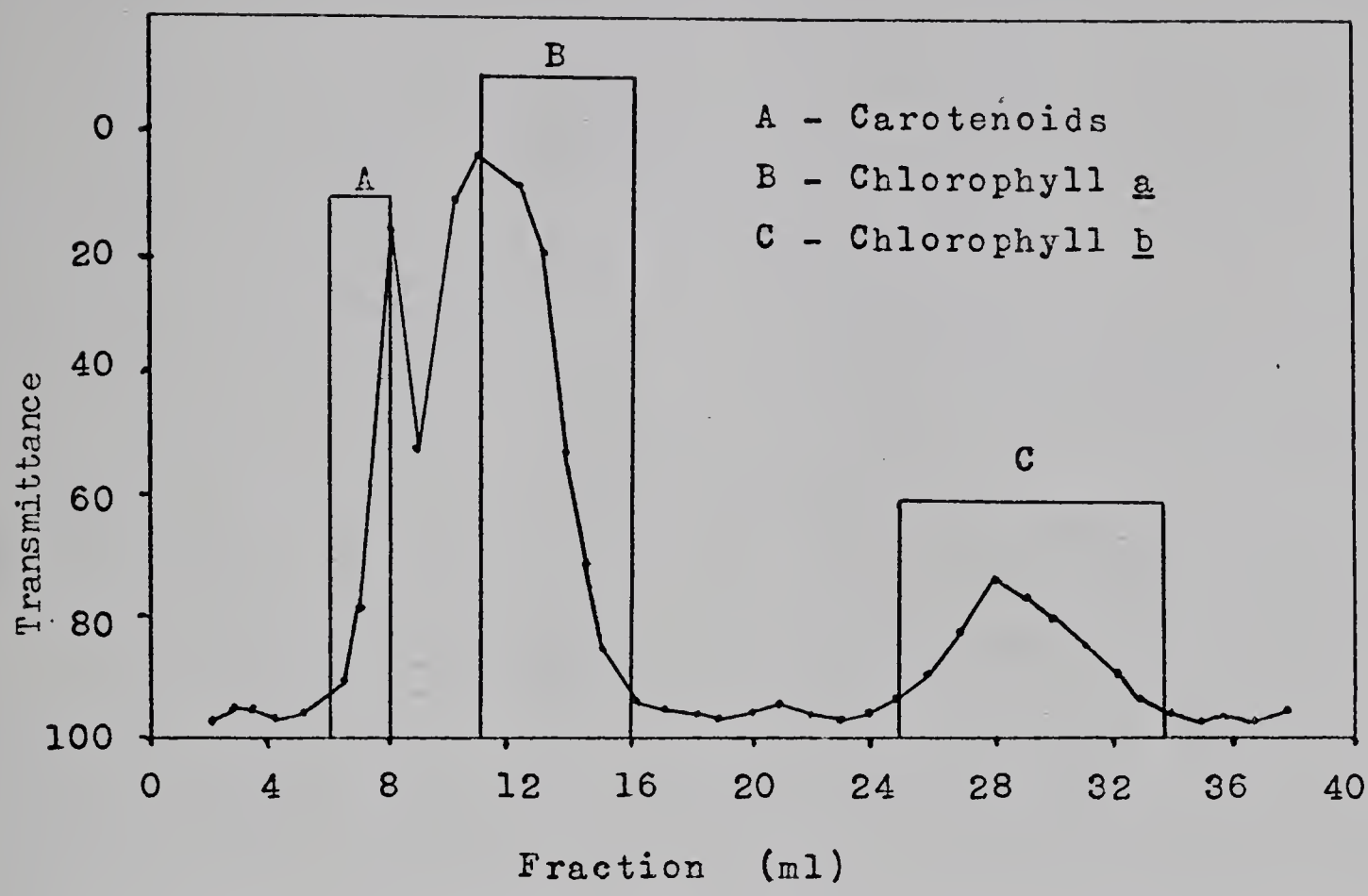


Figure 6

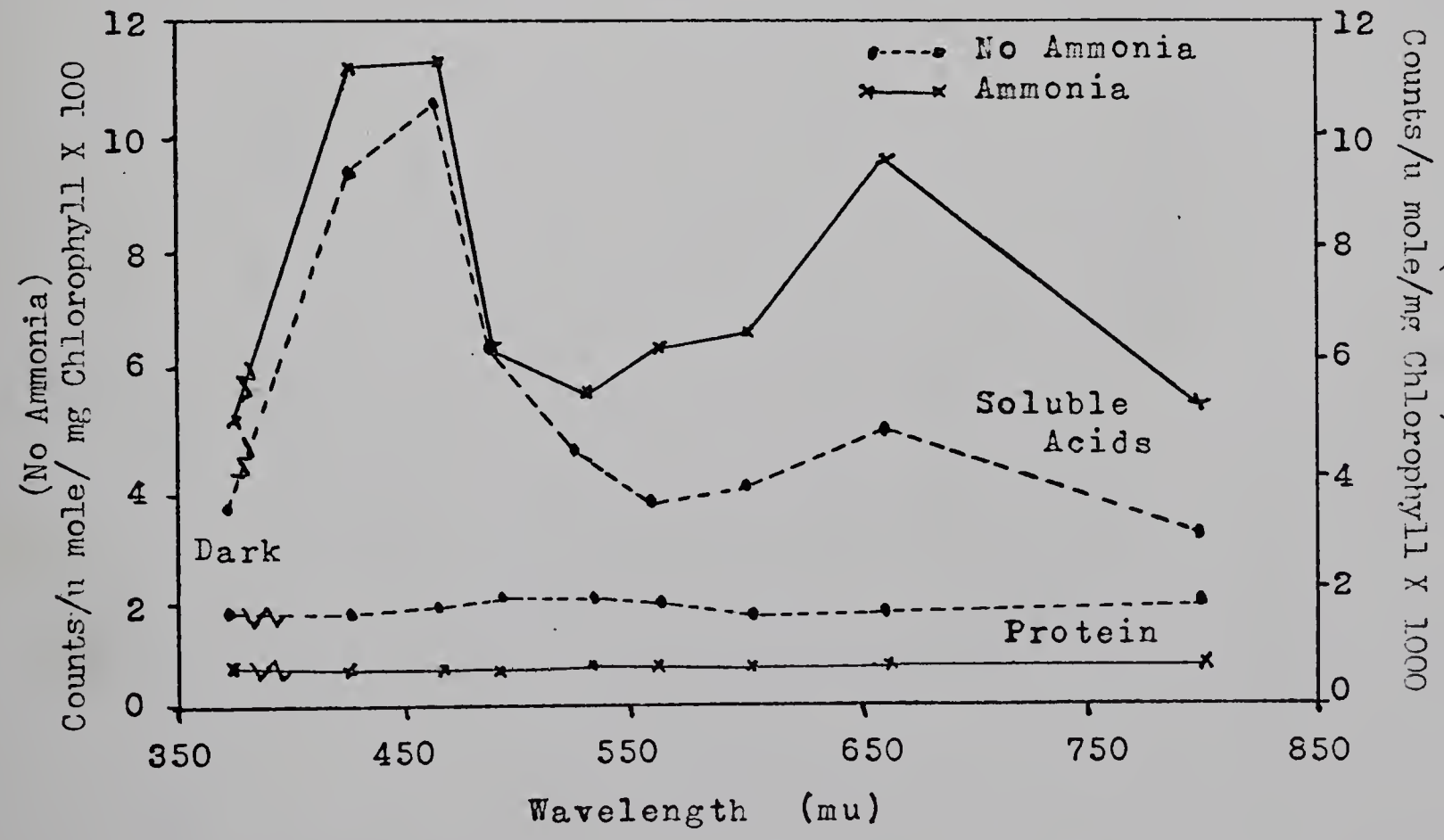


Fig. 5 Absorption spectra of individual pigments isolated from purified wax bean chloroplasts and separated on a sucrose column.

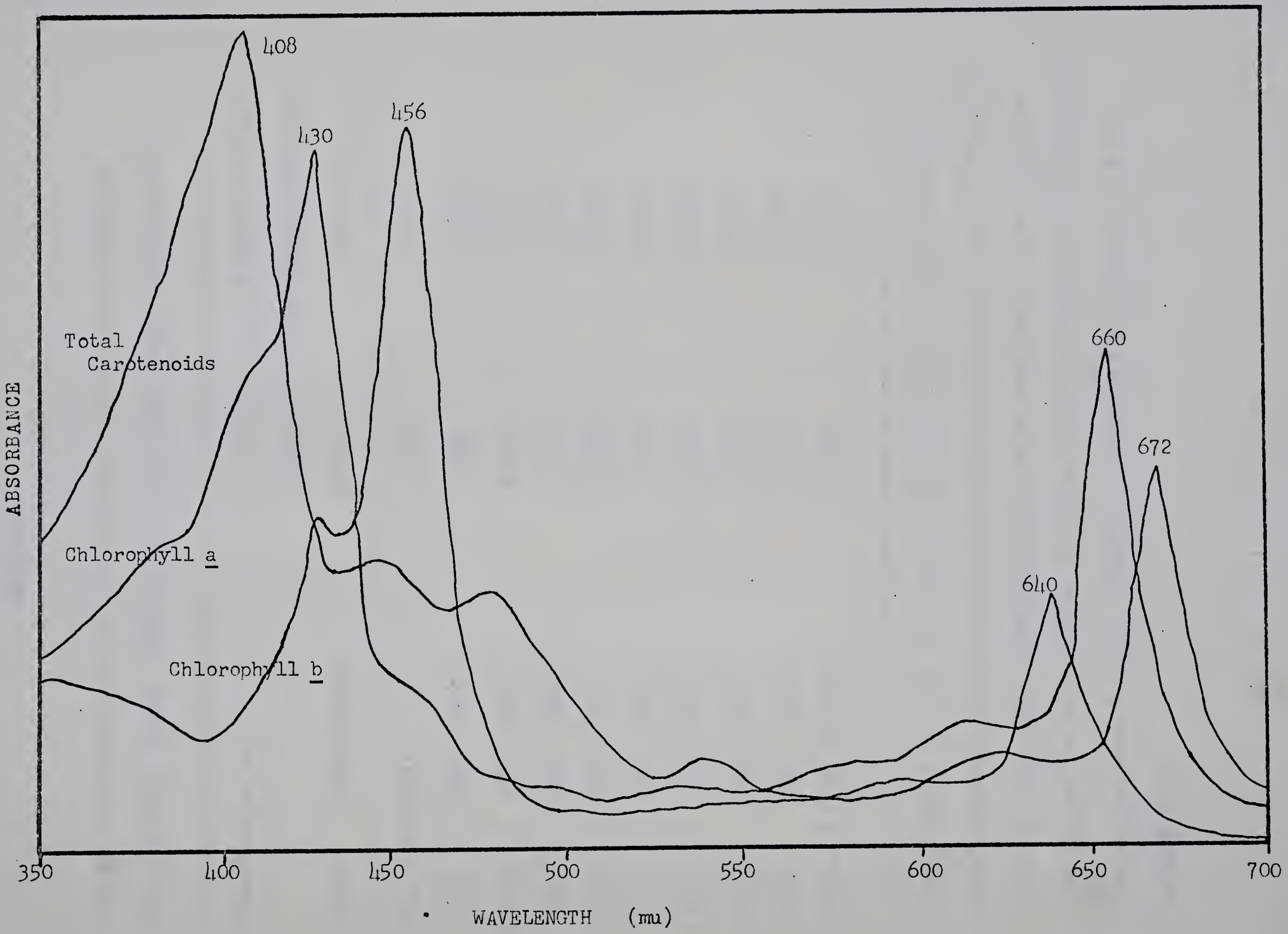

408
430
456
660
672
640
Total
Carotenoids
Chlorophyll a
Chlorophyll b
ABSORBANCE
350
400
450
500
550
600
650
700
WAVELENGTH (mu)

TABLE IV

Total Incorporation of ^{14}C without Ammonia into Soluble and Protein Amino Acids by Purified Wax Bean Chloroplasts.

Wavelength mu		Acidic and Neutral Acids Counts/u mole/mg Chlorophyll	
Peak	50% Transm.	Soluble	Protein
Dark Control		338	179
425	393 - 463	938	159
465	436 - 492	1065	170
493	460 - 532	623	194
532	500 - 577	420	188
560	540 - 605	338	192
600	580 - 655	389	162
660	630 - 675	486	162
800	720 - 900	296	179
White Light Control		625	172

The results are an average of 24 individual determinations. After separation into soluble and protein amino acid fractions 6 to 10 individual preparations were combined. The results are therefore an average of three separate amino acid analyses. The difference between counts of replicate analyses did not exceed 10%.

TABLE V

Total Incorporation of ^{14}C with Ammonia into Soluble and Protein Amino Acids by Purified Wax Bean Chloroplasts.

Wavelength mu		Acidic and Neutral Acids Counts/u mole/ mg Chlorophyll	
Peak	50% Transm.	Soluble	Protein
Dark Control		5197	693
425	393 - 463	11218	656
465	436 - 492	11291	653
493	460 - 532	6276	532
532	500 - 577	5443	650
560	540 - 605	6362	633
600	580 - 655	6550	627
660	630 - 675	9601	633
800	720 - 900	5248	644
White Light Control		9599	655

The results are an average of 18 individual determinations. After separation into soluble and protein amino acid fractions 6 to 10 individual preparations were combined. The results are therefore an average of three separate amino acid analyses. The difference between counts of replicate analyses did not exceed 10%.

as compared to its absence. The incorporation into the protein fraction was six times greater in the presence of ammonia. The total number of counts incorporated into the protein amino acid fraction remained essentially constant and at the same level as the dark control both in the presence and absence of ammonia.

Among the four individual protein amino acids into which most of the label was incorporated in the absence of ammonia (fig. 9, table VIII) the percentage of the total label in threonine and glutamic acid remained essentially constant and at the same level as the dark control. Aspartic acid showed a slight increase in incorporation in both the red (600 mu) and the blue (425 mu) regions. An increase in serine above that of the dark control occurred in the blue (425 mu) region.

Among the individual protein amino acids for experiments with ammonia present the percentage of total label incorporated remained constant in all the amino acids except threonine and serine (fig. 10, table IX). Serine showed an increase above the dark control at 425 mu, while threonine showed a decrease at this wavelength.

Although various amino acids of both the soluble and protein fractions became labeled, only those containing consistently more than 1% of the total label are reported. In some instances, labeling was detected for amino acids so small in quantity as to be insensitive to the ninhydrin test.

TABLE VI

Incorporation of ^{14}C without Ammonia into Individual Soluble Amino Acids by Purified Wax Bean Chloroplasts.

Wavelength mu		Amino Acids	
Peak	50% Transm.	% Aspartic	% Glutamic
Dark Control		6	47
425	393 - 463	9	43
465	436 - 492	14	59
493	460 - 532	5	37
532	500 - 577	6	50
560	540 - 605	6	50
600	580 - 655	6	40
660	630 - 675	6	46
800	720 - 900	6	47
White Light Control		6	67

The results are an average of 24 individual determinations. After separation into soluble and protein amino acid fractions 6 to 10 individual preparations were combined. The results are therefore an average of three separate amino acid analyses. The difference between counts of replicate analyses did not exceed 10%.

TABLE VII

Incorporation of ^{14}C with Ammonia into Individual Soluble Amino Acids by Purified Wax Bean Chloroplasts.

Wavelength mu		Amino Acids				
Peak	50% Transm.	% Asp.	% Thre.	% Ser.	% Glut.	% Gly
Dark Control		12	4	4	75	4
425	393 - 463	7	6	2	82	1
465	436 - 492	8	6	3	79	3
493	460 - 532	9	5	2	73	4
532	500 - 577	13	4	4	72	3
560	540 - 605	13	5	4	72	2
600	580 - 655	8	7	2	80	1
660	630 - 675	6	8	3	82	1
800	720 - 900	11	5	4	71	5
White Light Control		6	5	2	83	1

The results are an average of 18 individual determinations. After separation into soluble and protein amino acid fractions 6 to 10 individual preparations were combined. The results are therefore an average of three separate amino acid analyses. The difference between counts of replicate analyses did not exceed 10%.

Fig. 7 The percent incorporation of ^{14}C without ammonia into individual soluble amino acids by purified wax bean chloroplasts.

Fig. 8 The percent incorporation of ^{14}C with ammonia into individual soluble amino acids by purified wax bean chloroplasts.

Figure 7

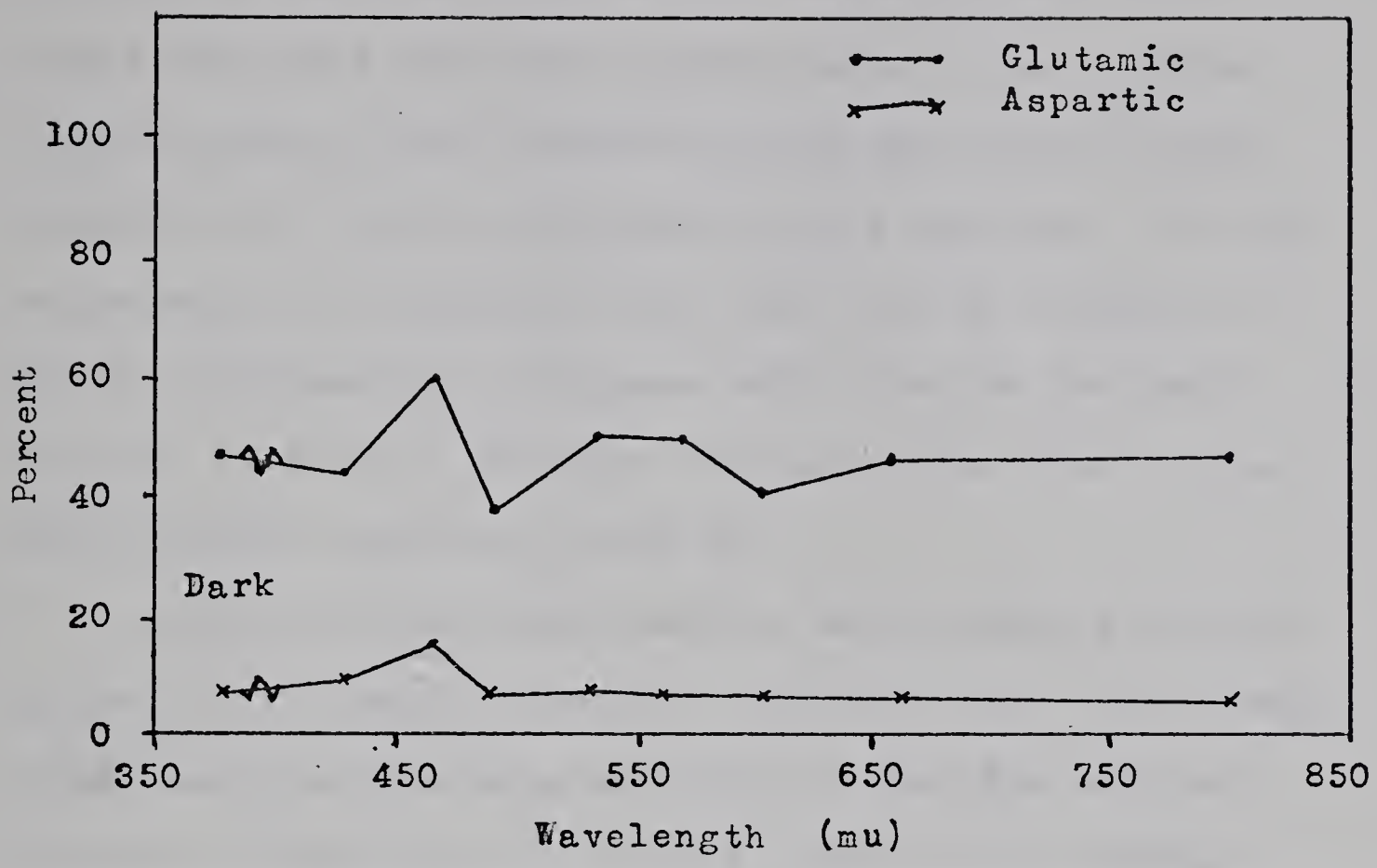
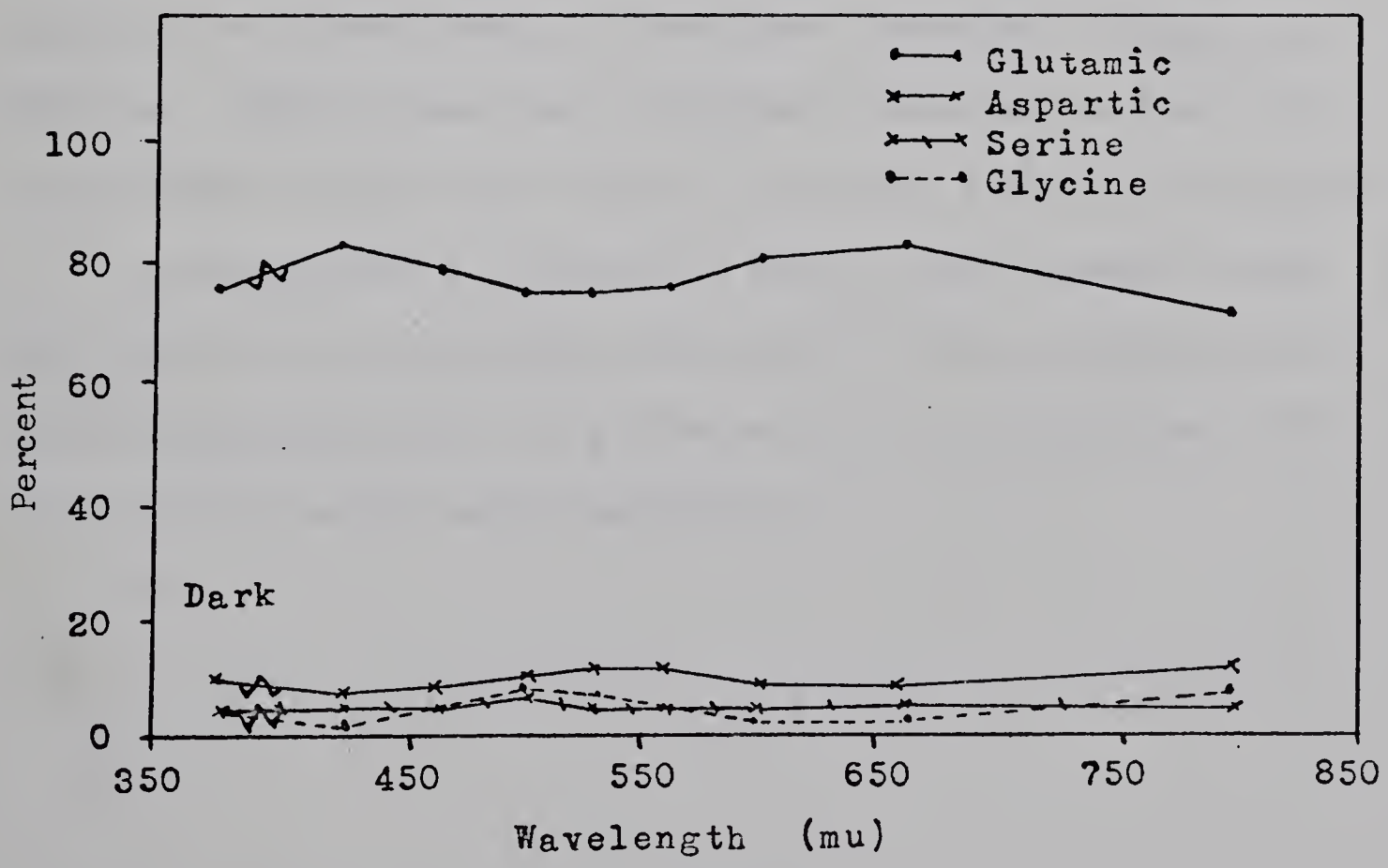


Figure 8



An increase in the incorporation of label, in the absence of ammonia, into soluble aspartic acid (fig. 7, table VI) above the dark control occurred in the blue region (460 mu) and remained at the same level as the dark control for the remainder of the spectrum. On the other hand, for glutamic acid there was an increase at 460 mu followed by a decrease below that of the dark control at 500 mu. Another decrease below that of the dark control occurred at 600 mu.

Among the individual soluble amino acids from experiments with ammonia present, glutamic acid showed small increases over the dark control for the blue and red regions of the spectrum (fig. 8, table VII), whereas aspartic acid had a slight decrease for the same two regions. Glycine also showed a decrease below the dark control in these areas. Threonine increased slightly at 660 mu. Serine remained relatively constant and at the same level as the dark control over the wavebands studied.

A comparison of figures 9 and 7, and figures 6 and 10, shows that variations observed for the soluble amino acid fractions were not reflected in the variations for the protein amino acid fractions.

TABLE VIII

Incorporation of ^{14}C without Ammonia into Individual Protein Amino Acids by Purified Wax Bean Chloroplasts.

Wavelength mu		Amino Acids			
Peak	50% Transm.	% Aspartic	% Thre.	% Ser.	% Glut.
Dark Control		28	11	8	14
425	393 - 463	39	10	21	11
465	436 - 492	32	12	9	13
493	460 - 532	33	9	9	14
532	500 - 577	32	8	11	13
560	540 - 605	30	10	8	12
600	580 - 655	37	10	7	12
660	630 - 675	30	7	8	11
800	720 - 900	29	12	9	12
White Light Control		34	11	9	10

The results are an average of 24 individual determinations. After separation into soluble and protein amino acid fractions 6 to 10 individual preparations were combined. The results are therefore an average of three separate amino acid analyses. The difference between counts of replicate analyses did not exceed 10%.

TABLE IX

Incorporation of ^{14}C with Ammonia into Individual Protein Amino Acids by Purified Wax Bean Chloroplasts.

Wavelength mu		Amino Acids					
Peak	50% Transm.	% Asp.	% Thre.	% Ser.	% Glut.	% Pro.	% Gly.
Dark Control		39	16	8	23	8	4
425	393 - 463	39	8	17	23	8	4
465	436 - 492	40	13	11	23	8	3
493	460 - 532	39	15	11	22	8	3
532	500 - 577	42	12	12	22	9	4
560	540 - 605	40	13	12	22	8	4
600	580 - 655	40	11	12	24	9	4
660	630 - 675	41	12	12	23	8	3
800	720 - 900	39	13	11	23	8	4
White Light Control		42	11	13	22	8	3

The results are an average of 18 individual determinations. After separation into soluble and protein amino acid fractions 6 to 10 individual preparations were combined. The results are therefore an average of three separate amino acid analyses. The difference between counts of replicate analyses did not exceed 10%.

Fig. 9 The percent incorporation of ^{14}C without ammonia into individual protein amino acids by purified wax bean chloroplasts.

Fig. 10 The percent incorporation of ^{14}C with ammonia into individual protein amino acids by purified wax bean chloroplasts.

Figure 9

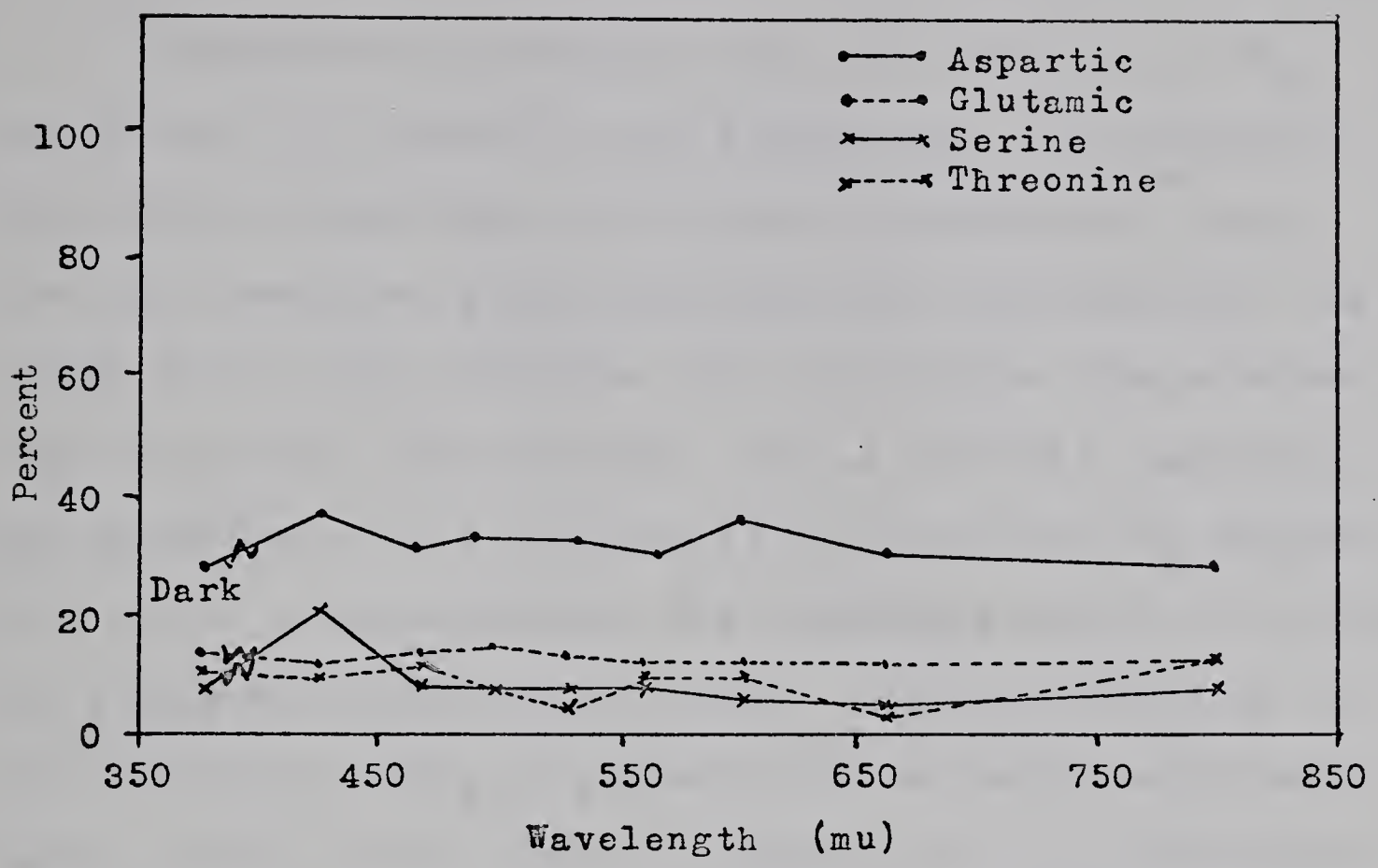
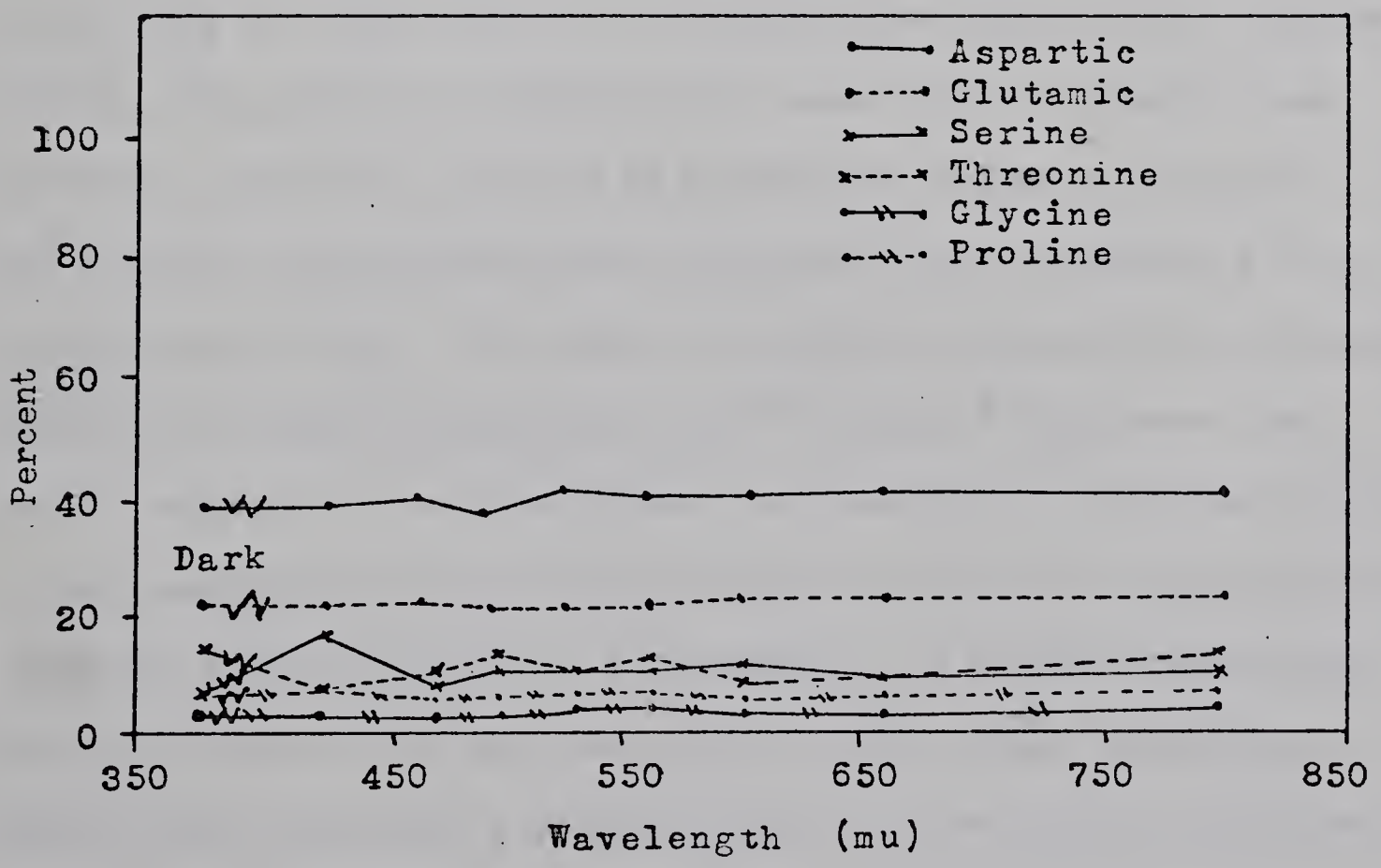


Figure 10



DISCUSSION

Photosynthetic mechanisms for the reduction of CO_2 are known to be seated in the chloroplast. The mechanism has three increasingly complex and interdependent steps; the Hill reaction, phosphorylation, and CO_2 reduction. In order for the CO_2 reduction step to function, the previous two steps must also function. For an isolated suspension of chloroplasts to be capable of incorporating CO_2 without the aid of added cofactors, the organelles must be preserved in a morphologically intact state. Further metabolism of the incorporated CO_2 to products such as amino acids would also require a high degree of intactness of the chloroplast in order to retain the necessary enzymes.

Chloroplast suspensions having the ability to incorporate CO_2 at high rates were prepared by Jensen and Bassham (29). The method of preparation used did not remove completely particles such as mitochondria which are capable of further metabolizing the intermediates produced by the reduction of CO_2. To study the effects of light of different quality on the incorporation of ^{14}C from $^{14}CO_2$ into the amino acids by the chloroplast, a homogenous purified chloroplast suspension from higher plants capable of photosynthesis without added cofactors is desirable. A method, employing first a buffer for the isolation of the crude chloroplast pellet and secondly a discontinous sucrose density gradient

centrifugation for purifying the crude chloroplast pellet, was developed. Figure 3 is a photograph showing the purity and intactness of the isolated chloroplast suspensions obtained by this method. The integrity of the isolated chloroplasts was evident for periods up to two hours.

The first purpose of these experiments was, as stated above, to develope a method for obtaining a purified chloroplast pellet capable of incorporation of $^{14}CO_2$ into amino acids without the addition of cofactors. The second purpose was to study the effects of different wavelengths of light on the amino acids labeled to see if the same labeling pattern would be obtained for isolated chloroplasts from higher plants as those obtained for algae (8, 21, 22, 42) and whole leaves or leaf discs (3, 10, 26, 46).

The difference in activity of the purified chloroplasts in incorporating ^{14}C into amino acids between white light and dark was substantial (tables IV and V). For experiments without ammonia, dark incorporation into soluble amino acids was 338 counts, while incorporation in light was 625 counts. With ammonia present, dark incorporation was 5197 counts while white light incorporation was 9599 counts. In both cases the effect of light was to approximately double the amount of label incorporated into the amino acids. As the only source of label was $^{14}CO_2$, photosynthesis must have taken place. The incorporation obtained in darkness may be explained by the presence of reduced compounds which

could have arisen from the brief exposure of the leaf material to light during the isolation procedure.

The possibility of bacteria having a large influence on these results was rejected for two reasons. Agar test plates of the solutions were prepared, but no bacteria were found to be present. Since the solutions contained no bacteria, the only source of contamination would be the plant material. Because of the short duration of the runs, and because of the nature of the labeled material in the substrate, the possibility of the activity having arisen from bacteria was highly unlikely.

The addition of ammonia to the reaction mixture increased the amount of incorporation of label into amino acids 15 times. Two possibilities for this effect of ammonia exist. One is that ammonia produces a direct stimulatory effect and the other is that the ammonia may become incorporated into the amino acids. Work on the incorporation of ^{15}N labled ammonia by Bassham and Kirk (8), Reisner et al. (40) and other workers (34, 47, 54) have shown that ^{15}N is incorporated into the amino acids, and that glutamic acid incorporated the highest amount of the label. It is therefore more likely that the large increase in labeling due to the addition of ammonia is a result of incorporation rather than a stimulatory effect.

When label was fed to the chloroplasts, a lag period of approximately 1/2 hour was observed before any label was

detected in the amino acids. This could be due to the activity of the label fed, and the carbonate concentration used. Ellyard and Gibbs (16) found that lag period recoveries were proportional to the bicarbonate concentration. This could be a partial explanation of the lag period observed as unlabeled CO_2 concentrations were of the order of 2 u moles per ml, while labeled CO_2 concentrations were only a very small fraction of a u mole. Another partial explanation may lie in the dilution of the incorporated label in all the intermediate pools in the pathways to amino acid synthesis.

According to the present concept of physics, the energy of radiation is contained in packets called quanta. The energy content of the quantum is inversely proportional to the wavelength of the light. The longer the wavelength of light the less energy contained per quantum. Therefore the energy content of a quantum of blue light is much higher than for a quantum of red light. With a given energy level, the quantum fluxes are directly proportional to the wavelength of the light. As a consequence, the adjustment of light intensity in all the cells to a uniform energy level resulted in the use of different quantum fluxes in the different cells.

Photosynthesis by whole leaves and Chlorella under different light qualities and tracing the $^{14}CO_2$ label in the amino acids has been studied by several workers (3, 22, 46).

Krotkov (32) found the specific activity of some amino acids produced during photosynthesis in the blue wavebands to be higher than those produced in the red bands. Cayle and Emerson (12) found the specific activity of the amino acids to be almost twice as great after photosynthesis in the blue as after photosynthesis in the red. The results shown in figure 6 and tables IV and V agree with those obtained by these workers. The flattening of the peak in the blue region of figure 6 (ammonia present) may be due to nitrogen starvation. The amount of ammonia fed was quite small. The difference between the 425 mu region and the 465 mu region in figure 6 is approximately 130 counts without ammonia and approximately 110 counts with ammonia . These two differences are of the same magnitude indicating that all the ammonia fed in the blue region may have been incorporated resulting in a depletion of ammonia to a level when ammonia was absent.

From figure 6 it may be noted that the 465 mu band incorporated ammonia and label at a higher rate than the 425 mu band. A possible explanation for this may be found from the work of Warburg et al. (52) with Chlorella. The action spectrum for blue light oxygen evolution was characterized by a sharp peak at 456 mu that fell away sharply to less than 50% effectiveness at 440 mu and 480 mu. Due to the width of the light band passed by the filters used in these experiments, the 425 mu filter included the wave-

bands from the area defined by Warburg. This may account for the high incorporation found in the 425 mu band.

Increases at different wavelengths for individual soluble and protein amino acids (figs. 7 and 9) would seem to indicate two different pathways of synthesis. Protein aspartic acid increased at two wavelengths, 425 mu and 600 mu, while soluble aspartic acid increased only at 465 mu. Protein serine showed a large increase at 425 mu, but soluble serine contained less than 1% of the total label incorporated. When ammonia was present in the reaction mixture (figs. 8 and 10), protein serine again showed an increase at 425 mu. Soluble glutamic acid showed two increases, one over the red region from 600 mu to 660 mu and the other at 425 mu. When ammonia was absent, soluble glutamic acid showed an increase only in the 465 mu band. Work by Andreeva and Korozheva (3) tend to confirm the trends in these results. They found that the type of light not only influenced the total rate of amino acid formation, but also changed the direction of amino acid synthesis. Krotkov (32) advanced two tentative theories to explain such differences in the influence of the wavelength of incident light. One theory states that one of the enzymes involved in the early stages of photosynthesis may have a pigment as its prosthetic group, which is activated by some particular wavelength. Depending on the form in which this enzyme is present, the flow of early CO_2 reduction intermediates is directed along different pathways. The other theory is based

on the relative importance of two photochemical acts responsible for different reductants and products. The spectral composition of the incident light determines the relative importance of these two photochemical acts and this in turn, by causing different reductants and products to appear, affects the path of carbon in photosynthesis. A third and similiar theory to explain the difference in the effects of light quality was given by Nichiporovich and Voskressenskaya (39). There may be in existence additional photochemical reactions possessing action spectra differing from those of the fundamental photochemical reactions of photosynthesis. The ratio between the fundamental and auxiliary photochemical reactions of the process of photosynthesis depends on the composition of the light. This would be one of the causes of variation in the composition of the direct products of photosynthesis under the action of long- or short-wave light bands.

The amount of the total label incorporated into soluble glutamic acid in the presence of ammonia was 75% to 80% (fig.8). Without ammonia added (fig. 7) approximately 50% of the total label incorporated appeared in soluble glutamic acid. Incorporation of label into the other individual acids for ammonia present and absent ranged from below 1% to 20% of the total. This may be explained by the findings of Bassham and Kirk (8). From their results the reductive amination to form glutamic acid seemed to be the primary

route for the incorporation of ammonia during the photosynthesis of amino acids. They found the relation between the time of maximum labeling of glutamic acid and the maximum rate of labeling of the other amino acids studied indicated that the amino groups in the other acids could all have arisen by transamination of their respective keto acids by glutamic acid. They concluded that there was no reason to think that amino acids other than glutamic acid incorporated ammonia by a direct reductive amination.

There are two other points to be considered. One is the difference in amounts of label incorporated into amino acids at different wavebands for soluble and protein amino acids. This would indicate different pathways and precursors of synthesis. Smith, Bassham and Kirk (42) have shown that there may be several pools of amino acids in plants. It is possible that different pools have different sources of material and pathways for their formation. Some of these pools may contribute amino acids for the synthesis of protein. The other point is that changes in the soluble amino acids were not reflected by parallel changes in the protein amino acids. The seemingly non light dependent incorporation of label into the protein amino acids is shown in figure 6. This occurred at a much lower rate than the incorporation of label into the soluble amino acids. From figures 9 and 10 it can be seen that formation of some individual protein amino acids is light dependent. Bamji and

Jagendorf (6) found that isolated wheat chloroplasts were able to incorporate amino acids without light if certain reduced cofactors were present. It would appear that the formation of the amino acids and reduced cofactors necessary for the formation of protein is light dependent, but that the direct formation of protein is not.

In order to determine whether one or all of the three major pigments, the carotenoids, chlorophyll a and chlorophyll b, might be directly linked to the degree of labeling observed in a particular amino acid, the pigments were isolated from the purified chloroplast and separated into the three classes. The absorption spectra of these separated pigments is shown in figure 5. The absorption spectra obtained for chlorophylls a and b in this experiment corresponds very well to those obtained by French (19). For chlorophyll a peaks occurred at 430 mu and 660 mu, and for chlorophyll b at 456 mu and 640 mu. A comparison of these spectra with the results given in figure 6 shows the major absorption areas of the pigments to occurr over the same wavebands as does ^{14}C incorporation. For individual soluble and protein amino acids the areas showing maximum incorporation again correspond to the major absorption areas of one of the pigments. However, considering that at 50% transmission, the band width of individual filters was approximately 70 mu, a feature inherent in the design of the filters, it becomes impossible to designate an individual pigment as responsible for the increase in photosynthetic products under certain filters.

CONCLUSION

In order to study the effects of light of different wavebands on the photosynthesis of amino acids, a series of light filters were constructed. As a consequence of adjusting the light intensities of all the filters to equal energies, different quantum fluxes were used in the different cells. Another limitation of the filters was that the maximum energy that could be obtained in the blue filter was 2090 ergs/cm^2/sec. Hence all the other cells had to be adjusted to this level.

Chloroplasts in a morphologically intact state were isolated from the leaves of Kinghorn wax bean and purified on a discontinous sucrose density gradient. The chloroplasts when incubated with $^{14}CO_2$ in white light incorporated twice as much label into the amino acids as the dark control. With ammonia present in the incubation mixture the amount of label incorporated into the amino acids under white light was increased 15 times. This increase was probably due to an incorporation of the ammonia into the amino acids rather than a stimulatory effect.

When the chloroplasts were incubated under light of different wavelengths, incorporation of label above that of the dark control occurred in the blue and the red regions. The wavebands most effective in incorporation of label into individual soluble amino acids were not the same as those

which were most effective for the individual protein amino acids. This might indicate different pools of the same amino acid arising from different pathways of synthesis.

Although absorption spectra of the separated pigments isolated from the purified chloroplasts were taken, no relation between any one pigment and the increase in the products of photosynthesis under a particular waveband could be made due to the approximate 70 mu band width passed by each of the filters at 50% transmission. However, the results show that the action spectrum for photosynthesis of amino acids by purified chloroplasts parallels the action spectrum for overall photosynthesis by higher plants. Moreover, it confirms that the chlorophylls and carotenoids are the major pigments involved.

Soluble glutamic acid contained up to 50% and 80% of the total label incorporated without and with ammonia respectively. The other individual amino acids were found to contain from less than 1% to 20% of the total label. This supports the theory that glutamic acid is the major route for the incorporation of ammonia into the plant.

The possibility of bacteria having a large effect on the results was rejected because of the nature of the labeled material, the short duration of the runs, and because of their absence from the buffer solutions used.

LITERATURE CITED

1. ANG, D.B. 1966. Effects of several spectral bands of visible light on development and lipid and protein metabolism in soybean seedlings. M.Sc. Thesis, Edmonton, Alberta.

2. ANDREEVA, T.F. 1956. Synthesis of proteins in green leaves. Fiziologiya Rastenii Akad. Nauk. SSSR 3: 157 - 163.

3. ANDREEVA, T.F. and KOROZHEVA, G.F. 1964. Influence of spectral composition of light and its intensity on the formation of amino acids in leaves. Fiziologiya Rastenii Akad. Nauk. SSSR. 11: 810 - 821.

4. ARNON, D.I., ALLEN, M.B. and WHATELY, F.R. 1956. Photosynthesis by isolated chloroplasts. (IV) General concept and comparison of three photochemical reactions. Biochim. Biophys. Acta. 20: 449 - 461.

5. BAKER, J.E. and THOMPSON. J.F. 1961. Assimilation of ammonia by nitrogen starved cells of Chlorella vulgaris. Plant Physiol. 36: 208 - 212.

6. BAMJI, M.S. and JAGENDORF, A.T. 1966. Amino acid incorporation by wheat chloroplasts. Plant Physiol. 41: 764 - 770.

7. BASSHAM, J.A., MORAWIECKA, B. and KIRK, M. 1964. Protein synthesis during photosynthesis. Biochim. Biophys. Acta. 90: 542 - 552.

8. BASSHAM, J.A. and KIRK, M. 1964. Photosynthesis of amino acids. Biochim. Biophys. Acta. 90: 553 - 562.

9. BASSHAM, J.A. 1965. Photosynthesis, the path of carbon. In Plant Biochemistry. Edited by J. Bonner and J.E. Varner. Academic Press. New York and London. 875 - 902.

10. BENNO, P. 1964. Protein synthesis in green leaves. I. Protein synthesis in various subcellular fractions following $^{14}CO_2$ photosynthesis. Z. Naturforsch. 19b: 235 - 248.

11. BORTHWICK, H.A. and HENDRICKS, S.B. 1960. Photoperiodism in plants. Science. 132: 1223 - 1225.

12. CAYLE, T. and EMERSON, R. 1957. Effect of wavelength on the distribution of carbon-14 in the early stages of photosynthesis. Nature. 179: 89 - 90.

13. CHAMPIGNY, M.L. 1956. Incorporation of $^{14}CO_2$ in amino acids of leaves of Kalanchoe diagremontiana in photosynthesis. Compt. Rend. Acad. Sci. 243: 83 - 85. Chem. Abst. 50: 17015g.

14. DEKEN-GRENSON, M. de 1954. Grana formation and synthesis of chloroplastic proteins induced by light in portions of etiolated leaves. Biochim. Biophys. Acta. 14: 203 - 211.

15. EISENSTADT, J.M. 1965. Protein synthesis in chloroplasts and chloroplast ribosomes in Biochemistry of Chloroplasts. Advanced study institute held at Aberystwyth August 1965. Academic Press. New York and London. 341 - 349.

16. ELLYARD, P.W. and GIBBS, M. 1967. The effect of oxygen on photosynthesis in chloroplasts. Plant Physiol. 42: S-33.

17. EMERSON, R. 1957. Dependence of yield of photosynthesis in long-wave red on wavelength and intensity of supplementary light. Science. 125: 746.

18. FLETCHER, R.A. 1964. Photomorphogenetic effects of regions of the visible spectrum on certain plant species. Ph.D. Thesis. Edmonton, Alberta.

19. FRENCH, C.S. 1960. The chlorophylls in vivo and in vitro. Encyclopedia of Plant Physiol. 5: 252 - 297.

20. GREGORY, F.G. and SEN, P.K. 1937. Physiological studies in plant nutrition. VI. The relation of respiration rate to the carbohydrate and nitrogen metabolism of the barley leaf as determined by nitrogen and potassium defficiency. Ann. Bot. (london) N.S. 1: 521 - 561.

21. HAUSCHILD, A.H.W., NELSON, C.D. and KROTKOV, G. 1961. Effect of light of different quality on the products of photosynthesis in Chlorella vulgaris. Plant Physiol. 36: XXVI.

22. HAUSCHILD, A.H.W., NELSON, C.D. and KROTKOV, G. 1962. The effect of light quality on the products of photosynthesis in Chlorella vulgaris. Can. J. Bot. 40: 179 - 189.

23 HAUSCHILD, A.H.W., NELSON, C.D. and KROTKOV, G. 1962. The effect of light quality on the products of photosynthesis in green and blue-green algae and in photosynthetic bacteria. Can. J. Bot. 40: 1619 - 1630.

24. HAXO, V. 1960. The wavelength dependence of photosynthesis and the role of accessory pigments. In Comparative Biochemistry of Photoreactive Systems. Edited by M.B. Allen. Academic Press. New York and London. 339 - 360.

25. HEBER, V. 1962. Protein synthesis in chloroplasts during photosynthesis. Nature. 195: 91 - 92.

26. HELLEBUST, J.A. and BIDWELL, R.G.S. 1963. Sources of carbon for the synthesis of protein amino acids in attached photosynthesizing wheat leaves. Can. J. Bot. 41: 985 - 994.

27. HENDRICKS, S.B. and BORTHWICK, H.A. 1959. Photocontrol of plant development by the simultaneous excitation of two interconvertable plant pigments. Proc. Nat. Acad. Sc. U.S. 45: 344 - 349.

28. HILLER, R.G. 1964. Studies on the incorporation of $^{14}CO_2$ into glutamic acid in Chlorella pyrenoidosa. Phytochem. 3: 569.

29. JENSEN, R.G. and BASSHAM, J.A. 1966. Photosynthesis by isolated chloroplasts. Proc. Nat. Acad. Sc. 56: 1095 - 1101.

30. KOK, B. 1965. Photosynthesis: The path of energy. In Plant Biochemistry. Edited by J. Bonner and J.E. Varner. Academic Press. New York and London. 903 - 960.

31. KROTKOV, G. 1960. The nature of photosynthetic products. Trans. Royal Soc. Can. III. 54: 1 - 14.

32. KROTKOV, G. 1964. The influence of the wavelength of incident light on the path of carbon in photosynthesis. Trans. Royal Soc. Can. IV. 2: 205 - 215.

33. MACKINNEY, G. 1941. Absorption of light by chlorophyll solutions. J. Biol. Chem. 140: 315.

34. MAC VICAN, R. and BURRIS, R.H. 1948. Studies on nitrogen metabolism in tomatoes with use of isotopically labeled ammonium sulfate. J. Biol. Chem. 176: 511 - 516.

35. MARGULIES, M. and PARENTI, F. 1967. In vivo synthesis of soluble and lamellar chloroplast proteins. Plant Physiol. 42: S-33.

36. Mc LEOD, G.C. and FRENCH, C.S. 1960. The function of various plant pigments in photosynthesis. Carnegie Instit. of Washington Year Book. 336 - 339.

37. MILLER, R.A. 1965. Physiological and biochemical studies of a mutant from Gateway barley. Ph.D. Thesis. Edmonton, Alberta.

38. NICHIPOROVICH, A.A. 1956. Tracer atoms used to study the products of photosynthesis depending on conditions under which the process takes place. Proc. Inter. Conf. Peaceful uses of Atomic Energy, Geneva. 12: 340 - 346.

39. NICHIPOROVICH, A.A. and VOSKRESENSKAYA, N. 1959. Effect of radiation of various wavelength regions on plant composition. Proc. IX Inter. Bot. Cong. 2: 281 - 282.

40. REISNER, G.S., GERING, R.K. and THOMPSON, J.F. 1960. Metabolism of nitrate and ammonia by Chlorella. Plant Physiol. 35: 48 - 52.

41. SAGER, R. and ISHIDIA, M.R. 1963. Chloroplast DNA in Chalmydomonas. Proc. Nat. Acad. Sc. 50: 725.

42. SMITH, D.C., BASSHAM, J.A. and KIRK, M. 1961. Dynamics of photosynthesis of carbon compounds. II. Amino Acid synthesis. Biochim. Biophys. Acta. 48: 299 - 313.

43. SMITH, J.H. and BENITZ, A. 1955. Modern Methods of Plant Analysis. Edited by K Paech and M.V. Tracey. Springer Verlag. Berlin.

44. STEWARD, F.C., BIDWELL, R.G.S. and YEMM, E.W. 1958. Nitrogen metabolism, respiration and growth of cultured plant tissue. J. Exp. Bot. 9: 285 - 305.

45. THOMPSON, J.F. and STEWARD, F.C. 1952. The analysis of the alcohol insoluble nitrogen of plants by quantitative procedures based on paper chromatography. J. Exp. Bot. 3: 170 - 187.

46. TREGUNNA, E.B., KROTKOV, G. and NELSON, C.D. 1962. Effect of white light red light and blue light on the nature of the products of photosynthesis in tobacco leaves. Can. J. Bot. 40: 317 - 326.

47. VICKERY, H.B., PUCHER, G.W., SCHOENHEIMER, R. and RITTENBERG, G. 1940. The assimilation of ammonia by the tobacco plant. A preliminary study with isotopic nitrogen. J. Biol. Chem. 135: 531 - 539.

48. VOSKRESENSKAYA, N.P. 1950. Effect of wavelength on synthesis of carbohydrates and protein in a leaf. Doklady Akad. Nauk. SSSR. 72: 173 - 176. Chem. Abst. 44: 10057a.

49. VOSKRESENSKAYA, N.P. 1953. Effect of illumination on the nature of photosynthetic products. Fiziologiya Rastenii Akad. Nauk. SSSR. 8: 42 - 56.

50. VOSKRESENSKAYA, N.P. 1956. Synthesis of organic and amino acids during photosynthesis under various conditions of illumination. Fiziologiya Rastenii Akad. Nauk. SSSR. 3: 49 - 57.

51. VOSKRESENSKAYA, N.P. and GRISHINA, G.S. 1956. Utilization of $^{14}CO_2$ by plants under various conditions of illumination. Doklady Akad. Nauk. SSSR. 106: 256 - 268. Chem Abst. 50: 10197i.

52. WARBURG, O., KRIPPAHL, G., SCHRODER, W. , BACHHOLZN, W. and THIEL, E. 1956. Action of weak blue-green light on the quantum yield in photosynthesis. Z. Naturforsch. 9b: 164 - 165. Chem. Abst. 48: 10145c.

53. WALKER, D.A. 1967. Biochemistry of Chloroplasts. Proc. Nato Advanced Study Instit. Aberystwyth, August 1965. Academic Press. New York and London. 341 - 349.

54. YEMM, E.W. and WILLIS, A.J. 1956. The respiration of barley plants. IX. The metabolism of roots during the assimilation of nitrogen. New Phytol. 55: 229 - 252.

55. ZAK, E.G. and NICHIPOROVICH, A.A. 1963. The pathways of amino acid synthesis during photosynthesis. Fiziologiya Rastenii Akad. Nauk. SSSR. 11: 805 - 809.

56. ZALIK, S. and MILLER, R.A. 1960. Construction of large low cost filters for plant growth studies. Plant Physiol. 35: 696 - 699.

APPENDIX

0.33M Sucrose Phosphate Buffer.

This buffer is a modification of that used by Walker (53). All quantities are for two litres of solution. The mixed and adjusted solution was filtered through coarse sintered glass into polyethylene storage bottles.

Sucrose	225.6 grams
KCl	1.492 grams
$MgCl_2 \cdot 7H_2O$	2.020 grams
KH_2PO_4	27.20 grams
K_2HPO_4	34.80 grams
pH	6.80

Phosphate Buffer

All quantities are for one litre of solution. The mixed solution was filtered through coarse sintered glass into a 1 litre glass storage bottle.

KH_2PO_4	13.60 grams
K_2HPO_4	17.40 grams
pH	6.80

Density Gradients

The gradient solutions are a modification of those used by Sager and Ishida (41). All quantities are for 500 ml of solution. The mixed solution was filtered through coarse sintered glass into polyethylene storage bottles.

1.0M (a)	Sucrose	171.2 grams
1.5M (b)	Sucrose	256.8 grams
2.0M (c)	Sucrose	342.4 grams
	KCl	0.373 grams
	$MgCl_2 \cdot 7H_2O$	0.505 grams
	KH_2PO_4	0.680 grams
	K_2HPO_4	0.870 grams
	pH	6.80

Ninhydrin Reagent

Preparation of 4N sodium propionate buffer.

Sodium propionate	384.3 g/1000 ml
propionic acid	93.0 ml 1000 ml

4N Sodium prop. buffer	1000 ml
Methyl Cellosolve	3000 ml
Ninhydrin	80 grams
Total	4000 ml

Elution Buffers for Amino Acid Analysis

pH	2.2	3.25	4.25	5.28
Use	Samp. Dil. Buffer	Col. 1a 1b Eluent	Col. 1a 1b Eluent	Col. 2 Eluent
Na Conc.	0.2N	0.2N	0.2N	0.35N
Citric H_2O	21 g	840 g	840 g	491 g
NaOH (97%)	8.4 g	330 g	330 g	288 g
Conc. HCl	16 ml	426 ml	188 ml	136 ml
Caprylic	0.1 ml	4.0 ml	4.0 ml	2.0 ml
Thiodiglycol	20 ml	200 ml	200 ml	----
BRIJ-35 sol.	2.0 ml	80 ml	80 ml	40 ml
Final Volume	1 litre	40 litres	40 litres	20 litres